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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12Q 1/68	A1	(11) International Publication Number: WO 98/37243
		(43) International Publication Date: 27 August 1998 (27.08.98)

(21) International Application Number: PCT/US98/08896

(22) International Filing Date: 2 January 1998 (02.01.98)

(30) Priority Data:
60/034,410 2 January 1997 (02.01.97) US

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(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: Z–CHROMOSOMAL MARKERS DERIVED FROM CHICKEN (GALLUS DOMESTICUS) AND USE THEREOF IN CHROMOSOMAL MAPPING

(57) Abstract

We have developed a chicken (*Gallus domesticus*) Z–chromosome–specific DNA library in a phage vector, by means of chromosome microisolation and microcloning. The chromosomal origin, specificity and purity was evaluated by fluorescent *in situ* hybridization (FISH) on chicken metaphases. Heterologous chromosome painting, using this Z–chromosome–specific probe on turkey (*Meleagris gallopavo*) metaphases identified its homologous Z–chromosome, under the same stringent conditions as that used in the chicken, indicating a high degree of Z–chromosome sequence homology among these two species. This chicken Z–chromosome library will facilitate the development of Z–chromosome–specific DNA markers that will be useful for genetic mapping in the domestic chicken and related avian species. The Z–chromosome–specific DNA probe will also be useful for studies pertaining to the sex chromosome evolution in avian species.

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INTERNATIONAL SEARCH REPORT

Int. ional Application No
PCT/US 98/08896

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
A	LEVIN I ET AL: "Genetic map of the chicken Z chromosome using random amplified polymorphic DNA (RAPD) markers." GENOMICS, (1993 APR) 16 (1) 224-30, XP002067078 cited in the application see the whole document ---	1-7
A	WO 94 07907 A (ZOOGEN INC) 14 April 1994 see the whole document ---	1-7
A	WO 96 39505 A (ISIS INNOVATION ;GRIFFITHS RICHARD (GB); TIWARI BELA (GB)) 12 December 1996 see the whole document ---	1-7

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 June 1998	18/06/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Molina Galan, E

1

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Int. ..tional Application No
PCT/US 98/08896

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BRUFORD M W ET AL: "Minisatellite DNA markers in the chicken genome. II. Isolation and characterization of minisatellite loci." ANIMAL GENETICS, (1994 DEC) 25 (6) 391-9, XP002067079	
A	BIOLOGICAL ABSTRACTS, vol. 95, Philadelphia, PA, US; abstract no. 423269, PONCE DE LEON F A ET AL: "Analysis of the chicken NM7659 T(Z;1) translocation with chromosome painting probes and GBP banding." XP002067083 & EIGHTY-FOURTH ANNUAL MEETING OF THE POULTRY SCIENCE ASSOCIATION, INC., EDMONTON, ALBERTA, CANADA, AUGUST 14-18, 1995. POULTRY SCIENCE 74 (SUPPL. 1). 1995. 9. ISSN: 0032-5791,	
A	BIOLOGICAL ABSTRACTS, vol. 95, Philadelphia, PA, US; abstract no. 423268, AMBADY S ET AL: "A Z - chromosome specific DNA library." XP002067084 & EIGHTY-FOURTH ANNUAL MEETING OF THE POULTRY SCIENCE ASSOCIATION, INC., EDMONTON, ALBERTA, CANADA, AUGUST 14-18, 1995. POULTRY SCIENCE 74 (SUPPL. 1). 1995. 8. ISSN: 0032-5791,	
A	PONCE DE LEÓN ET AL.: "Development of a bovine X chromosome linkage group and painting probes to asses cattle, sheep and goat X chromosome segment homologies" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 93, April 1996, WASHINGTON US, pages 3450-3454, XP002067080 cited in the application	
P,X	AMBADY S ET AL: "Development of a chicken Z - chromosome -specific DNA library." JOURNAL OF HEREDITY, (1997 MAY-JUN) 88 (3) 247-9, XP002067081 see the whole document	1-7

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1

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	ZIMMER R ET AL: "Generation of chicken Z - chromosome painting probes by microdissection for screening large-insert genomic libraries." CYTOGENETICS AND CELL GENETICS, (1997) 78 (2) 124-30. XP002067082 see the whole document ---	1-7
P,X	BIOLOGICAL ABSTRACTS, vol. 97, Philadelphia, PA, US: abstract no. 487182, PONCE DE LEON F A ET AL: "Chicken genome project: Chromosome-specific libraries and applications of genome scans to assess genomic variation." XP002067085 see abstract & REVISTA BRASILEIRA DE REPRODUCAO ANIMAL 21 (3). 1997. 102-105. ISSN: 0102-0803. -----	1-7

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 98/08896

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9407907 A	14-04-1994	CA 2124220 A	14-04-1994
		AU 662564 B	07-09-1995
		AU 2696092 A	26-04-1994
		EP 0623139 A	09-11-1994
WO 9639505 A	12-12-1996	AU 5906996 A	24-12-1996
		EP 0832218 A	01-04-1998

PATENT COOPERATION TREATY

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NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

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in its capacity as elected Office

Date of mailing: 27 August 1998 (27.08.98)	
International application No.: PCT/US98/08896	Applicant's or agent's file reference: 002076-001
International filing date: 02 January 1998 (02.01.98)	Priority date: 02 January 1997 (02.01.97)
Applicant: PONCE DE LEON, F., Abel et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International preliminary Examining Authority on:

30 July 1998 (30.07.98)

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2. The election [X] was

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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
002076-001		
International application No.	International filing date (day/month/year)	Priority date (day/month/year)
PCT/US 98/ 08896	02/01/1998	02/01/1997
International Patent Classification (IPC) or national classification and IPC		
C12Q1/68		
Applicant		
UNIVERSITY OF MASSACHUSETTS et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 4 sheets, including this cover sheet.

☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consists of a total of _____ sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
30/07/1998	15. 10. 98
Name and mailing address of the IPEA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk - Netherlands Tel.: (+31-70) 340-2040, Tx. 31 651 epo nl Fax: (+31-70) 340-3016	E. MOLINA GALAN Telephone No. (+31-70) 3403560

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US98/08896

I. Basis of the report

1. This report has been drawn up on the basis of *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.)*

☒ the international application as originally filed

☐ the description, pages , as originally filed
pages , filed with the demand
pages , filed with the letter of

☐ the claims. Nos. , as originally filed
Nos. , as amended under Article 19
Nos. , filed with the demand
Nos. , filed with the letter of

☐ the drawings, sheets / fig. , as originally filed
sheets / fig. , filed with the demand
sheets / fig. , filed with the letter of

2. The amendments have resulted in the cancellation of:

☐ the description, pages:

☐ the claims. Nos.

☐ the drawings, sheets / fig.

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2 (c)).

4. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty	Claims	1- 7	YES
	Claims		NO
Inventive Step	Claims	1- 7	YES
	Claims		NO
Industrial Applicability	Claims	1- 7	YES
	Claims		NO

2. Citations and Explanations

2.1 CITATIONS

Reference is made to the following documents:

D1: Genomics, 16, 1993, 224- 230, Levin et al.
D2: Proc. Natl. Acad. Sci., 93, 1996, 3450- 3454, Ponce de León et al.

2.2 NOVELTY (Art. 33(2) PCT)

2.2.1 The present application does satisfy the criterion set forth in Article 33(2) PCT because the subject- matter of Claims 1- 7 is new in respect of prior art as defined in the regulations (Rule 64(1)- (3) PCT).

2.3 INVENTIVE STEP (Art. 33(3) PCT)

2.3.1 Document D1, which is considered to represent the most relevant state of the art, discloses (cf. discussion) DNA markers derived from the chicken Z chromosome and methods for using them. The subject- matter of Claim 1 differs in that different markers are claimed.

2.3.2 The problem to be solved by the present invention may therefore be regarded as the

provision of alternative DNA markers derived from the chicken Z chromosome. The solution would be the markers identified by Seq. Ids. 1- 19.

2.3.3 Although D2 discloses a method to derive markers from chromosomes similar to that used in the application, it does not seem obvious to derive exactly the markers claimed by the applicant, specially taking into account that the source is a complete chromosome which has not been completely sequenced.

2.3.4 For these reasons the markers claimed can not be regarded as a simple choice and the IPEA is of the opinion that the present application satisfies the criterion set forth in Article 33(3) PCT and the subject- matter of claims 1- 7 involves an inventive step (Rule 65(1)(2) PCT).

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REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

09/108896

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International Application No. PCT/US97/23822

International Filing Date (02.01.98) 02 JAN 1998

Name of receiving Office and "PCT International Application" PCT INTERNATIONAL APPLICATION RO/US

Applicant's or agent's file reference *(if desired) (12 characters maximum)* 002076-001

Box No. I TITLE OF INVENTION

Z-CHROMOSOMAL MARKERS DERIVED FROM CHICKEN (GALLUS DOMESTICUS) AND USE THEREOF IN CHROMOSOMAL MAPPING

Box No. II APPLICANT

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (i.e. country) of residence if no State of residence is indicated below.)*

UNIVERSITY OF MASSACHUSETTS
A Public Institution of Higher Education of the Commonwealth of Massachusetts, as Represented by its Amherst Campus
Office of Vice Chancellor for Research at Amherst
Amherst, Massachusetts 01002
United States of America

☐ This person is also inventor.

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State *(i.e. country)* of nationality: US

State *(i.e. country)* of residence: US

This person is applicant for the purposes of ☐ all designated States ☒ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

BOX No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (i.e. country) of residence if no State of residence is indicated below)*

PONCE DE LEON, F. Abel
134 Wildflower Drive
Amherst, Massachusetts 10002
United States of America

This person is:

☐ applicant only

☒ applicant and inventor

☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality: US

State *(i.e. country)* of residence: US

This person is applicant for the purposes of ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

☒ Further applicants and/or (further) inventors are indicated on a continuation sheet.

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The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: ☒ agent ☐ common representative

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

TESKIN, Robin L.
BURNS, DOANE, SWECKER & MATHIS, L.L.P.
P.O. Box 1404
Alexandria, Virginia 22313-1404
United States of America

Telephone No. (703) 836-6620

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Teleprinter No.

☐ Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

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Continuation of Box No. III FURTHER APPLICANTS AND/OR (FURTHER) INVENTORS

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CIUFO, Stacy
56 Chesterfield Road
Amherst, Massachusetts 01002
United States of America

This person is:
- [ ] applicant only
- [X] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality: US

State *(i.e. country)* of residence: US

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- [ ] all designated States except the United States of America
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- [ ] the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (i.e. country) of residence if no State of residence is indicated below.)*

ROBL, James
196 Old Enfield
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United States of America

This person is:
- [ ] applicant only
- [X] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality: US

State *(i.e. country)* of residence: US

This person is applicant for the purposes of
- [ ] all designated States
- [ ] all designated States except the United States of America
- [X] the United States of America only
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Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (i.e. country) of residence if no State of residence is indicated below.)*

AMBADY, Sakthikumar

Kerala State
India

This person is:
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- [X] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality: IN

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Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (i.e. country) of residence if no State of residence is indicated below.)*

SMYTH, J. Robert, Jr.

Amherst, Massachussetts 01002
United States of America

This person is:
- [ ] applicant only
- [X] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality: US

State *(i.e. country)* of residence: US

This person is applicant for the purposes of
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PCT/US 97/23822

98/08896

Box No. V DESIGNATION OF STATES

The following designations are hereby made under Rule 4.9(a)*(mark the applicable check-boxes; at least one must be marked)*:

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- [X] **FI** Finland
- [X] **GB** United Kingdom
- [X] **GE** Georgia
- [X] **GH** Ghana
- [X] **HU** Hungary
- [X] **ID** Indonesia
- [X] **IL** Israel
- [X] **IS** Iceland
- [X] **JP** Japan
- [X] **KE** Kenya
- [X] **KG** Kyrgyzstan
- [X] **KP** Democratic People's Republic of Korea
- [X] **KR** Republic of Korea
- [X] **KZ** Kazakhstan
- [X] **LC** Saint Lucia
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- [X] **LU** Luxembourg
- [X] **LV** Latvia
- [X] **MD** Republic of Moldova
- [X] **MG** Madagascar
- [X] **MK** The former Yugoslav Republic of Macedonia
- [X] **MN** Mongolia
- [X] **MW** Malawi
- [X] **MX** Mexico
- [X] **NO** Norway
- [X] **NZ** New Zealand
- [X] **PL** Poland
- [X] **PT** Portugal
- [X] **RO** Romania
- [X] **RU** Russian Federation
- [X] **SD** Sudan
- [X] **SE** Sweden
- [X] **SG** Singapore
- [X] **SI** Slovenia
- [X] **SK** Slovakia
- [X] **SL** Sierra Leone
- [X] **TJ** Tajikistan
- [X] **TM** Turkmenistan
- [X] **TR** Turkey
- [X] **TT** Trinidad and Tobago
- [X] **UA** Ukraine
- [X] **UG** Uganda
- [X] **US** United States of America
- [X] **UZ** Uzbekistan
- [X] **VN** Viet Nam
- [X] **YU** Yugoslavia
- [X] **ZW** Zimbabwe

Check-boxes reserved for designating States (for the purposes of a national patent) which have become party to the PCT after issuance of this sheet:

- [X] GM Gambia
- [X] GW Guinea-Bissau
- [ ]

In addition to the designations made above, the applicant also makes under Rule 4.9(b) all designations which would be permitted under the PCT except the designation(s) of _____.

The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. *(Confirmation of a designation consists of the filing of a notice specifying that designation and the payment of the designation and confirmation fees. Confirmation must reach the receiving Office within the 15-month time limit.)*

PRIOUS 97/23821
98/28856

Box No. VI PRIORITY CLAIM Further priority claims are indicated in the Supplemental Box ☐

The priority of the following earlier application(s) is hereby claimed:

Country *(in which, or for which, the application was filed)*	Filing Date *(day/month/year)*	Application No.	Office of filing *(only for regional or international application)*
item (1) US	02 January 1997 (02.01.97)	60/034,410	
item (2)			
item (3)			

Mark the following check-box if the certified copy of the earlier application is to be issued by the Office which for the purposes of the present international application is the receiving Office (a fee may be required):

☒ The receiving Office is hereby requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) identified above as item(s): (1)

Box No. VII INTERNATIONAL SEARCHING AUTHORITY

Choice of International Searching Authority (ISA) *(If two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used):* ISA / EP

Earlier search *Fill in where a search (international, international-type or other) by the International Searching Authority has already been carried out or requested and the Authority is now requested to base the international search, to the extent possible, on the results of that earlier search. Identify such search or request either by reference to the relevant application (or the translation thereof) or by reference to the search request.*

Country (or regional Office) | Date *(day/month/year)*: | Number:

Box No. VIII CHECK LIST

This international application contains the following number of sheets:

1. request	:	4	sheets
2. description	:	14	sheets
3. claims	:	1	sheets
4. abstract	:	1	sheets
5. drawings	:	4	sheets
Total	:	24	sheets

This international application is accompanied by the item(s) marked below:

1. ☐ separate signed power of attorney
2. ☐ copy of general power of attorney
3. ☐ statement explaining lack of signature
4. ☐ priority document(s) *(identified in Box No. VI as item(s))*:
5. ☒ fee calculation sheet
6. ☐ separate indications concerning deposited microorganisms
7. ☐ nucleotide and/or amino acid sequence listing (diskette)
8. ☒ other *(specify)*: (Transmittal Letter and Receipt Card)

Figure No. _____ of the drawings (if any) should accompany the abstract when it is published.

Box. No. IX SIGNATURE OF APPLICANT OR AGENT

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).

Robin L. Teskin

For receiving Office use only (02.01.98)

1. Date of actual receipt of the purported international application: 76 Rec'd PCT/PTO 02 JAN 1998
2. Drawings: ☐ received: ☐ not received:
3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:
4. Date of timely receipt of the required corrections under PCT Article 11(2):
5. International Searching Authority specified by the applicant: ISA/EP
6. ☐ Transmittal of search copy delayed until search fee is paid

For International Bureau use only

Date of receipt of the record copy by the International Bureau: 10 FEBRUARY 1998 (10.02.98)

Z-CHROMOSOMAL MARKERS DERIVED FROM CHICKEN (GALLUS DOMESTICUS) AND USE THEREOF IN CHROMOSOMAL MAPPING

Field of the Invention

The invention relates to novel chromosomal markers derived from chicken and use thereof.

Background of the Invention

Livestock genome maps have progressed very rapidly in the past few years due to the availability of highly polymorphic DNA markers. But in many species, the maps are not dense enough to facilitate a thorough search for quantitative trait loci (QTLs). This is especially true in the case of the chicken. The chicken haploid karyotype consists of 39 chromosomes that are classified into two categories - the macrochromosomes and the microchromosomes. The largest five pairs of macrochromosomes and the Z-chromosome represent about 55 percent of the total DNA content of the chicken genome. The Z-chromosome covers about 210 cM of the estimated 2500 - 3,000 cM of the chicken genome map (Levin et al. *Genomics*, 16:224-230 (1993)).

Knowledge of the genetic composition of the chicken Z-chromosome is limited, in spite of the fact that this chromosome has the most detailed linkage map for this species, largely generated by classical linkage test analyses (Bitgood and Somes, *Poultry Breeding and Genetics*, 2nd Ed., Crawford RD, ed., Amsterdam: Elsevier, pp. 469-495 (1990)). To date, 19 known loci and 14 genetic markers consisting of 3 chicken middle repetitive sequence element (CRI) markers, 8 random amplified polymorphic DNA (RAPD) markers and 3 microsatellites have been assigned to the chicken Z-chromosome (Bitgood and Somes, (Id.) (1990); Saitoh et al, *Chrom. Res.*, 1: 239-251 (1993); Cheng et al, *Poultry Sci.*, 74: 1855-1874 (1995)).

The avian sex chromosome constitution differs from that of mammals because females are heterogametic (ZW) and males homogametic (ZZ). It has been observed from comparative linkage analyses that some of the sex linked

genes in mammals are autosomal in chicken, while some of the sex linked genes in chicken are autosomal in mammals (Bitgood and Somes, (Id.) (1990)). Accordingly, obtaining further information concerning the Z-chromosome of chickens would be beneficial in identifying sex-linked genes in chickens and related species.

Brief Description and Objects of the Invention

Thus, it is an object of the invention to identify novel chromosomal markers from the Z-chromosome of chicken. It is further an object of the invention to use such markers to construct a Z-chromosome specific DNA map and to use such chromosomal markers to identify Z-chromosome homologs in related avian species, e.g., turkey.

In order to develop a dense genetic map for chicken, it is important to generate a large number of polymorphic markers per chromosome (Cheng et al, *Poultry Sci.*, 741:1855-1874 (1995)). One way of achieving this goal is to develop chromosome-specific libraries. Chromosome flow-sorting has been the method of choice for the generation of chromosome-specific libraries in humans (Fuscoe et al, *Cytogenet Cell Genet*, 43:79-86 (1986)) and in swine (Langford et al, *Anim. Genet*, 24: 261-267 (1993)). Development of flow-sorted chromosomes is technically demanding and frequently yield preparations which have some degree of contamination with other chromosomes (Hozier and Davis, *Anal. Biochem*, 200: 205-127 (1992)).

A more effective and direct way of generating chromosome-specific DNA libraries is by chromosome microisolation and microcloning of the chromosome of interest. Chromosome specific libraries generated by chromosome microisolation have been used in swine (Ambady et al, (unpublished data)), cattle (Ponce de León et al, *Proc. Natl. Acad. Sci., USA*, (in press) 1996)), and chicken (Li et al, *Proc. of the 10th Eur. Colloq. on Cytogenetics of Domestic Animals*, Utrecht Univ., The Neth., p. 11, August 18-21 (1992)) genetic mapping studies in order to develop maps for particular chromosomes.

Generation of polymorphic markers from chromosome-specific libraries for all of the 8 pairs of the chicken macrochromosomes will enable saturation of about 55-70% of the chicken genome. Chromosome-specific DNA can also be used as heterologous chromosome painting probes in closely and distantly related species for comparative genome analysis, study of chromosomal evolution, and for identifying gross chromosomal abnormalities.

This application, in particular, provides a chicken Z-chromosome-specific DNA library, Z-chromosomal markers and use thereof as probes to identify the Z-chromosome homolog in related species, such as turkey.

Brief Description of the Figures

Figure 1 shows amplification of microsatellite markers by PCR and identification of polymorphisims.

Figure 2 shows a genetic map constructed using the identified microsatellite markers.

Figure 3 shows dinucleotide repeats present in the identified microsatellite markers.

Detailed Description of the Invention

Microisolation and microcloning:

Chicken metaphases were prepared from chicken fibroblast cultures following standard procedures, fixed briefly for 5 minutes each in 9:1, 5:1 and 3:1 methanol:acetic acid and dropped on clean coverslips. Chromosome microisolation and cloning was performed following the procedure described by Ponce de León et al (*Proc. Natl. Acad. Sci., USA* (in press) (1996)). Briefly, twelve copies of the chicken Z-chromosome were microisolated and transferred to clean siliconized coverslips. Proteinase-K digestion, phenol-chloroform extraction, *Sau*3AI (50U/μl, New England Biolabs) digestion and ligation to custom prepared *Sau*3AI adaptors were performed in a nanoliter drop. Ligation

products were digested with BglI enzyme (Promega, 10 units/μl) to cleave off the adaptor dimers that form during the ligation process.

The ligation product was PCR amplified and 10 μl of the amplified product was run on an agarose gel to determine the size of the amplified products. A 2 μl volume of this original amplification was labeled by PCR, using biotin-16-dUTP (Boehringer Mannheim). The purity, specificity and origin of the DNA fragments was determined by FISH on chicken metaphases following the procedure described by Ponce de León et al (*Proc. Natl. Acad. Sci. USA* (in press) (1996)). The remainder of the PCR product was digested with *Sau*3AI and passed through a Microcon 30 (Amicon Inc.) spin column to cleave and remove the flanking adaptors respectively.

In order to produce a chicken Z-chromosome-specific phage library, the digested DNA was cloned in a lambda ZAP Express vector (Stratagene) and packaged using Gigapack II Gold packaging extract (Stratagene). The library was amplified by plate lysate method following the manufacturer's protocol and stored at -70°C in 7% DMSO and 0.3% chloroform. Average size of library inserts was determined by PCR amplification of 30 randomly picked clones using the T3 and T7 priming sites flanking the insert.

Fluorescent in situ hybridizations

The Z-chromosome-specific DNA fragments were fluorescently labeled by PCR with biotin-16-dUTP (3:1 ratio of dTTP:biotin-16-dUTP) and passed through a Sephadex G-50 column to remove unincorporated nucleotides. The protocol described by Ponce de León (*Proc. Natl. Acad. Sci., USA* (in press) (1996)) was followed. Briefly, 200 nanograms of labeled Z-chromosome specific DNA was mixed with 6 μg of chicken competitor DNA (average size 200-400 bp) and 5.8 μg of salmon sperm DNA (average size 200-400 bp), precipitated and resuspended in 12 μl of hybridization buffer consisting of 50% deionized formamide, 1X SSC and 100% dextran sulphate to achieve a final DNA concentration of 1 μg/μl. The hybridization mix was denatured at 75°C for 5

minutes and reannealed at 37°C for 10 minutes and deposited on denatured (70% formamide, 2X SSC at 70°C for 2 minutes) chicken or turkey metaphases, mounted, sealed with rubber cement and incubated in a humidified chamber at 37°C for 18 to 20 hours. The slides were washed in 50% formamide/2X SSC at 42°C for 15 minutes and 0.1X SSC at 60°C for 15 minutes. Blocking was done using 2% blocking reagent (Boehringer Mannheim) and the signals were detected using avidin-FITC (5 μg/ml, Vector labs) in 1% blocking solution. Slides were washed in 4X SSC/0.1% Tween-20 for 15 minutes at 42°C, stained for 10 minutes in propidium iodide (400 ng/ml in 2X SSC) and rinsed for 5 minutes in 2X SSC/0.01% Tween-20. Slides were mounted in p-phenylenediamine-11 (PPD-11) antifade and observed under a Zeiss Axioskop fluorescent microscope.

Results

A chicken Z-chromosome specific DNA cocktail was developed by chromosome microisolation, *Sau*3AI digestion, adaptor ligation and PCR amplification. The amplified DNA fragments ranged in size from 400 bp to 1600 bp with the bulk of the DNA in the 500-1000 bp range. The origin, specificity and purity of the chromosomal DNA fragments was verified by FISH after PCR labeling of a small fraction of the DNA cocktail. The probes showed specific hybridization signal on a medium sized submetacentric chromosome identified as the Z-chromosome based on its morphology and G-banding pattern. After having confirmed the origin and purity of the preparation, the adaptors flanking the inserts were removed by *Sau*3AI digestion and column purification. Cloning was performed using equimolar ratios of the inserts to the vector ends (lambda ZAP Express, Stratagene). The original library consisted of a total of 8.48×10^5 plaques representing about 14 chicken Z-chromosome equivalents. The final titer of the amplified library was 1.2×10^{12} pfu/ml.

Thirty random plaques were selected and the inserts PCR-amplified using the T3/T7 priming sites flanking the inserts. The average insert size was about 1,000 bp (data not shown). This library was screened to identify microsatellite

containing clones to increase the marker density of the chicken Z-chromosome genetic linkage map.

Heterologous painting of turkey metaphase chromosomes:

The labeled chicken Z-chromosome-specific DNA fragments were used to perform FISH analysis on turkey metaphase chromosomes following the procedure described previously. Washes at the same stringency showed strong hybridization signals on a medium-sized submetacentric chromosome in turkey metaphases (data not shown). This chromosome was identified as the Z-chromosome homolog in the turkey. The obtained results indicate that the chicken and turkey Z-chromosome sequences are highly conserved. The red-legged partridge Z-chromosome has also been shown to be homologous to the chicken Z-chromosome (Dias el al, *Proc. of the XXIV Int. Cont. on Anim. Genet.*, Prague, Czech. p. 133 (July 23-24, 1994)). These results are similar to the FISH results obtained when the bovine X-chromosome painting probes were used on sheep and goat chromosomes (Ponce de León el al, *Proc. Natl. Acad. Sci., USA* (in press) (1996)) and with human X-chromosome probes on a wide range of mammalian species (Schertan el al, *Nat. Genet.*, 6:342-347 (1994)) indicating the high degree of sex chromosome conservation among all the mammalian species studied. Solinas-Toldo et al (*Genomics*, 27: 489-496 (1995)) have previously shown that human chromosome-specific painting probes could identify chromosomal segments in bovine that are homologous to specific human chromosomes. It is expected based on our results that chicken chromosome painting probes can similarly be used in closely and distantly related avian species to identify gross chromosomal rearrangements such as translocations and duplications that have occurred during avian evolution. Since the chicken Z-chromosome sequences are highly conserved in the turkey, the chicken Z-chromosome-specific microsatellite markers should be particularly useful for genetic mapping in turkey.

Conclusions

Genetic and physical mapping of human and animal genomes has been greatly facilitated by the use of chromosome specific DNA libraries. Mapping with libraries specific to a chromosome or chromosomal region increases marker saturation by reducing the gaps resulting from a purely random shotgun approach. This study was undertaken to construct a genetic and physical map of microsatellites on the chicken Z chromosome. This chromosome is the fifth largest in the chicken genome, comprising about 8% of the total. Notwithstanding its size, very few microsatellites have been assigned to it. DNA originating from the chicken Z chromosome was previously isolated and reported. This was used to construct a small insert library in Lambda ZAP Express, representing 14 chromosome equivalents. This library was screened for microsatellites with an (AC) 12 oligo, and positive clones were isolated. Confirmation of the presence of the microsatellite, as well as its approximate location along the cloned fragment was accomplished by PCR amplification. Clones with adequate flanking regions were sequenced, and primers for 19 microsatellites were constructed. These primers were used to genotype individuals from the East Lansing Poultry Reference Population and a linkage map was constructed. Fourteen markers were scorable and polymorphic in this population. The resulting map contains 12 markers in two linkage groups spanning 90 Cm and two unlinked markers. The physical location of each marker was established by fluorescent *in situ* hybridization (FISH). Preliminary results with four markers allowed the assignment of one linkage group to the long arm of the Z chromosome, and one to the short arm.

The following nucleic acid sequences are microsatellite markers identified by the above methods. As discussed supra, these markers are useful for genetic mapping and for study of the sex chromosome structure in avian species. Also, such markers should enable the identification of genes encoding desirable traits, e.g., genes involved in growth rates, and for identifying sex-linked genotypes.

EXAMPLE

The specific Gallus domesticus microsatellite markers identified are set forth below. As noted, these DNA markers will be useful for genetic mapping of domestic chicken as well as related avian species and for studies pertaining to evolution of the sex chromosome in avian species.

SEQUENCE 1 (43. Seq)

1 gatcactttc cctaatattc ttgtgtttct tgtttgttga cctgtaatgc
1 agttctgagt tttggaaagg aactaattaa gaccagagga gagataattt
101 tcttttatca aaaaacaaac aaacaaacaa aaaaacgaat tcttaccact
151 ttacaaaaat tttccatttt gaaggccagt acagccatag cattcatcta
201 ctttttgctt tggat

SEQUENCE 2 (71. Seq)

1 gatcaggtgg cctgtagtag acaacaacaa caatggggtg ccctttgttg
51 ccttagtctc taactcgcac ccacacacac tttcaagttg cttgtggcca
101 ttcttcaggg acagttcttc acaatctatt cctttcctga tgtagaaggc
151 gtcacctcct cccctcctgc ctcgtttgtc ccttctaaac tgcaggtatt
201 agtattgata gctaaggtca agtcatggga accatctcac caggtttcag
251 tgttggcaac tatgttatgc tttcttagga gcatggtggt tccaactctt
301 ccctgcttat ttcccaagct gtgtgtgatg gtaggatagc attcaagtgg
351 gaggagccta tcggcttttt ggaggtactc ctaaatccct gatattcccc
401 tgattcccgt acttcttcct tgccaagggc ccgccaatgc atagttcaat
451 ttctcatgca gacgctaagg aaaggtggac cc

SEQUENCE 3 (80 Seq.)

1 gatcgtatgt attttttac ataggataga aaatggccaa taggaaataa
51 gacagtacag ctactaagaa agaaacacaa ttacacacac acacacacac
101 acacacacac acacatttga aaaacgcgct gcacagcagt gtgggtattt
151 tttcacaaga gagacacact ctacagtaca cagccagctc tactttgtcg
201 cacagtctca gtgtgtgttt gccaacagga cgcggttcac agggagatat
251 tgtcctcttg tgtgtgtgga gacacagaga cagag

SEQUENCE 4 (81. Seq)

1 gatcccctgg aggaagggca atggcaaccc actccagtat tcttgcctga
51 agaataccat ggtcagtttt gcctcctggg ctatagtcca tggggttgca
101 aagagtcagg catgactgag cgactctctc tctctctctc tctctctctc
151 acacacacac acacacacac acacacggcg tctctctctc tctctataca
201 tataggctgt gtgtctcgct attctcacat gagggaaact catatctagc
251 acgtggcaca aatattgttt gtggctctca caaaagacat gtgggcgcac
301 aaaggtcccc ccccggtgga tacancgcct tggtttttta taacccaagc
351 ctgtg

SEQUENCE 5 (131 Seq)

1 gatcacatat gtaaactagg gaattgcata ataagattaa atgtaggtgt
51 agaacgtggc atgaaggaag gtagaattag gtggtaccta tctcttctga
101 aacaaactga gaatcctact accaatcaac atattctaca taccacacac
151 acatttttc tcgagtaaaa tataaactaa tgagaaactt ccctag

SEQUENCE 6 (147. Seq)

1 gatcccaagc aacacatagn cagacaatca cacacacaca cacacacaca
51 cacacacaca cacacacaca cacatcctct ccccacaata catcccgaga
101 gggggggagag acactctctc tccctctcta taggggagac ccggagagct
151 ggctctgttg tctctctaca ccggacatac agtggagcac atctcacact
201 tgtgtctttg tctctctaca ccggacatac agtggagcac atctcacact
251 tgtgtctcta tctctccctg tccctgttga tccatctctc ttcacacatc
301 tctccagatc ttagcgctag agtctcctgt cttctctctg cgcaatttgt
351 gtgatagaga cacctgatat gttgtgtggg ggagacatct gtgtgtctct
401 gtgtcatccc agaggatttt tctctcccac acttagaggc cttctcaaga
451 gatgggaggt tttaatgggg tgtg

SEQUENCE 7 (166. Seq)

1 gatcattctt ctgtttccca ttctaatggg aattctccac acacacacac
51 acacacacac acacacacat cttcttcccc ttacatggaa aaaaatcctc
101 cacacccctg gacactgatt actctccctc ttcccagaga gagatc

SEQUENCE 8 (196. Seq)

1 gatcccctag agaagggaat ggctactcac tccagtattc ttgcctggag
51 aattccgtgg tcagaggagc ctggaaggct ataatccata gagtcgcaag
101 agtcagacag gactgagtga ctaacacaca catgcacaca cacacacaca
151 cacacacaca cttgctctag ggagaggcat agagatgtaa tctctcctaa
201 aatgggggtg gcgatggccc ctgcggccaa gtaatcgcca cacatgcgta
251 ttccccttaa gattgggtta ggcctccctt atgaggagag accagggaga
301 gaatgggctc tctctctctc tcactcccca accgagtaag tggtaaaaaa
351 ggttttcctg gattacaatt ttggtgttac agaattggaa aaaaatattt
401 ttggggctcc cccctcagtt ta

SEQUENCE 9 (199. Seq)

1 ctagcaaaaa cacccccaca agttatgaaa acaacggctt aatatagtaa
51 tgtgtgtgtg tgtgtgtgtg tgttgcacac cacagttttc tctgatactc
101 aaacctctct ctttctctac aggggccccc cataacacag cggctgagat
151 gtgtgacggg aaggcgtggc cttttacaca tttgtggtat ggtctgccaa
201 ggcccccctat tgcccccccac aactacggag atacactagg ggcgacccgc
251 aggcgcgcga cccccaggtg gggccccgag

SEQUENCE 10 (204. Seq)

1 ctttaggagg ttctctcgag taagcttttt ggatttcttt ggttcccaag
51 catcacatgg tacaggcagt cacacacaca cacatacaca cacacacaca
101 cacacacaca cactcctctc cccacaatac ataccgagag gggggagaga
151 cactctctct ccctctctat agggggagcc ccacagagct ggctctgttg
201 tctctctcca ccggacatac agtggagcac atctcacact tctgtctcta
251 tctctccctg cccctgtgac atccatctct cttcacacaa tctcacccag
301 gatcttagcg ctagagaccc cctgtccttc ttctcctggg gaaatttttt
351 gtggataaga gacacccgat atattggtgt gggggagaac atcttgtgag
401 gtctctgttg tgccatccca acaggaattt ttatctcccc cacaattaga
451 ggcccctcct caagagtgtg tgagggtt

SEQUENCE 11 (235. Seq)

1 gatcacagat gtatgtattt ttttacatag gatagaaaat ggacaatagg
51 aaataagaca gtacagctac taagaaagaa cccacattta cacacacaca
101 cacacacaca cacacacaca agtgtttaat ccgctgcaca gcattgtgga
151 catttttaca caagagagac acactctaca gtttgcgccc agctctag

SEQUENCE 12 (249. Seq.)

1 gatcattctt ctgtttccca ttctaatgga attctccaca cacacacaca
51 cacacacaca cacacactct tctttctcct gacatggaaa aatctccccc
101 acaccccggg acactgattt ctctccctct ccccaacact gtgagcaaga
151 ggagtttatt ttgtgtgtgt cactcttcca gggagagaga gatc

SEQUENCE 13 (258. Seq)

1 ctaggcatcg gttgggaggt ggtgagtaat tacttgtctg acattagtcc
51 tgtaacattg ggtgtgtgtg tgtgtgtgtg tgtgtattcc ccttgggaat
101 tggttttctc aaccacaagt tcttcttttt ttttttctc ccccctttc
151 ttctgaaaat aagtacttgg ggggtttccg ccccccccgg taaataaaat

SEQUENCE 14 (290. Seq)

1 ctagtggctc ccaagcaaca catagccaga caacacacac acacacacac
51 acacacacac acacacacac acacacactc ctctccccac aatacatccc
101 gagaggggggg agagacactc tctctccctc tctatagcgg gagccccaca
151 gagctggctc tgctgtctct ctacaccgga catacagtgg agcacatctc
201 acattcgtgt ctctatctct ccctgccccc ggtgacatac atctctcttc
251 acacatctca ccaggtctga gcgctagagt ctcctgtctt ctctctgcgc
301 aatatttgtg atagagacat ctgatatatt gtgtgtggga gacatcttgt
351 gagtctctgt gtgcatccca gaggattttt atctccccac actag

SEQUENCE 15 (309. Seq)

1 gatccatgaa aactttccga gttgtattgt ctaggtgaaa acacacacaa
51 acacacacac acacacacac acacaacagg gagatgagtc ttgcaagaga
101 ataggggaga gttatgtcac caagtctggt gaggtatata gcgtataggg
151 agccaacatg tcagacatct gatgtgctaa gattaacatt ttattttatt
201 taatgtgtga gatctcatat agcggctctt cttatatatg acgtctcgca
251 atgtctcttt atgtgtgtta ttctctgagc ccctgggaga tatctgtcat
301 cagagagaag agacatacac atacaggggt tatatatttt ctccctgtgt
351 gtggagatgg agggtatttt ggacaagctc aacactcatt ggctcccaga
401 gagagaaaag gagcaactgt tgcacccggg gctctgtagc tgggatc

SEQUENCE 16 (341. Seq)

1 caattgggta catctacctg gtaccccacc cgggtggaaa atcgcatggg
51 cccgcggcgg ttctaggaag tactctcgag aagcttttgg gttctttggg
101 tcccaagcag cacatggaca ggcaatcaca cacacacaca cacacacaca
151 cacacacaca cacacacaca ctcctctccc cacaatacat cccgagaggg
201 gggagagtca ctctctctcc ctctctatag ggggcgcccc taagagctgg
251 ctctgttgtc tatctacacc gcacatacaa tggagcacaa ctcacactag

SEQUENCE 17 (398. Seq)

1 gatcaaagca tggaggtcat gccaggcact gaacaaaatg gtagagagtg
51 attctatgac tgactaagac ctcatgcaac aacaagtgaa gagtcacaac
101 tgcaaacaga agtacaactt agcaaatcct attttcagga aacactaaac
151 cgtaatactt gcacgatttt ttctttaata cagtaataat tcttttagaa
201 tttggatata tcttttaaga tacatatttg tctaaatacc aaggcaggat
251 atgagcataa aatagctaag gttagctatg gtgttatatt taagaagacc
301 acagagcaat aggagcatac ttttcttggg gtagaagggg cccttaaagg
351 tcacctag

SEQUENCE 18 (420. Seq)

1 ctagccacat cctataactc cactccacct ttaatcctga tttctgtgtc
51 tcttctctaa cctctatggc ctttctctaa agttccccaa tatcaacaat
101 ccttttcccc actgggacct ccagtttatt gattctacca tgtcactatc
151 catggtcaac cacttgtggt attataggat gtcgcgtgtg tgtgtgtgtg
201 tgtgtgcatg tgtgtgtgct tgggtgtcag agagttccaa tctgggggac
251 ctatggtttg taaacaacag gtctcttgcc aaggaagat

SEQUENCE 19 (435. Seq)

1 ctagcgctcg tgcccctgca gttcgacact cagtggctcc tccacacaca
51 cacacacaca cacatcaata tatatataga tagatagata gatagaggag
101 caatataagt ggcttctcta tttccagcat gttttgaaga gcataaactc
151 aacagagtat atataaatct gatgtgaccc atgtcatctg ctacagcatg
201 agaggggtta gtgatc

CLAIMS:

1. A Z-chromosomal marker DNA selected from the group consisting of Sequence I (43. Seq), Sequence 2 (71. Seq), Sequence 3 (80. Seq), Sequence 4 (81. Seq), Sequence 5 (131. Seq), Sequence 6 (147. Seq), Sequence 7 (166. Seq), Sequence 8 (196. Seq), Sequence 9 (199. Seq), Sequence 10 (204. Seq), Sequence 11 (235. Seq), Sequence 12 (249. Seq), Sequence 13 (258. Seq), Sequence 14 (290. Seq), Sequence 15 (309. Seq), Sequence 16 (341. Seq), Sequence 17 (398. Seq), Sequence 18 (420. Seq), and Sequence 19 (435. Seq).

2. A Z-chromosomal DNA library that contains at least one DNA sequence according to Claim 1.

3. A method of using at least one Z-chromosomal DNA according to Claim 1 for genetic mapping.

4. The method of Claim 3, wherein the genetic mapping is effected to construct a Z-chromosome specific DNA map.

5. The method of Claim 3, wherein the Z-chromosome DNA map is that of an avian species selected from the group consisting of chicken, turkey, partridge, duck, guinea hen, and goose.

6. The method of Claim 4, which is used to identify gross chromosomal rearrangements.

7. The method of Claim 6, wherein said chromosomal rearrangement comprises a translocation, deletion or duplication.

ABSTRACT

We have developed a chicken (*Gallus domesticus*) Z-chromosome-specific DNA library in a phage vector, by means of chromosome microisolation and microcloning. The chromosomal origin, specificity and purity was evaluated by fluorescent *in situ* hybridization (FISH) on chicken metaphases. Heterologous chromosome painting, using this Z-chromosome-specific probe on turkey (*Meleagris gallopavo*) metaphases identified its homologous Z-chromosome, under the same stringent conditions as that used in the chicken, indicating a high degree of Z-chromosome sequence homology among these two species. This chicken Z-chromosome library will facilitate the development of Z-chromosome-specific DNA markers that will be useful for genetic mapping in the domestic chicken and related avian species. The Z-chromosome-specific DNA probe will also be useful for studies pertaining to the sex chromosome evolution in avian species.

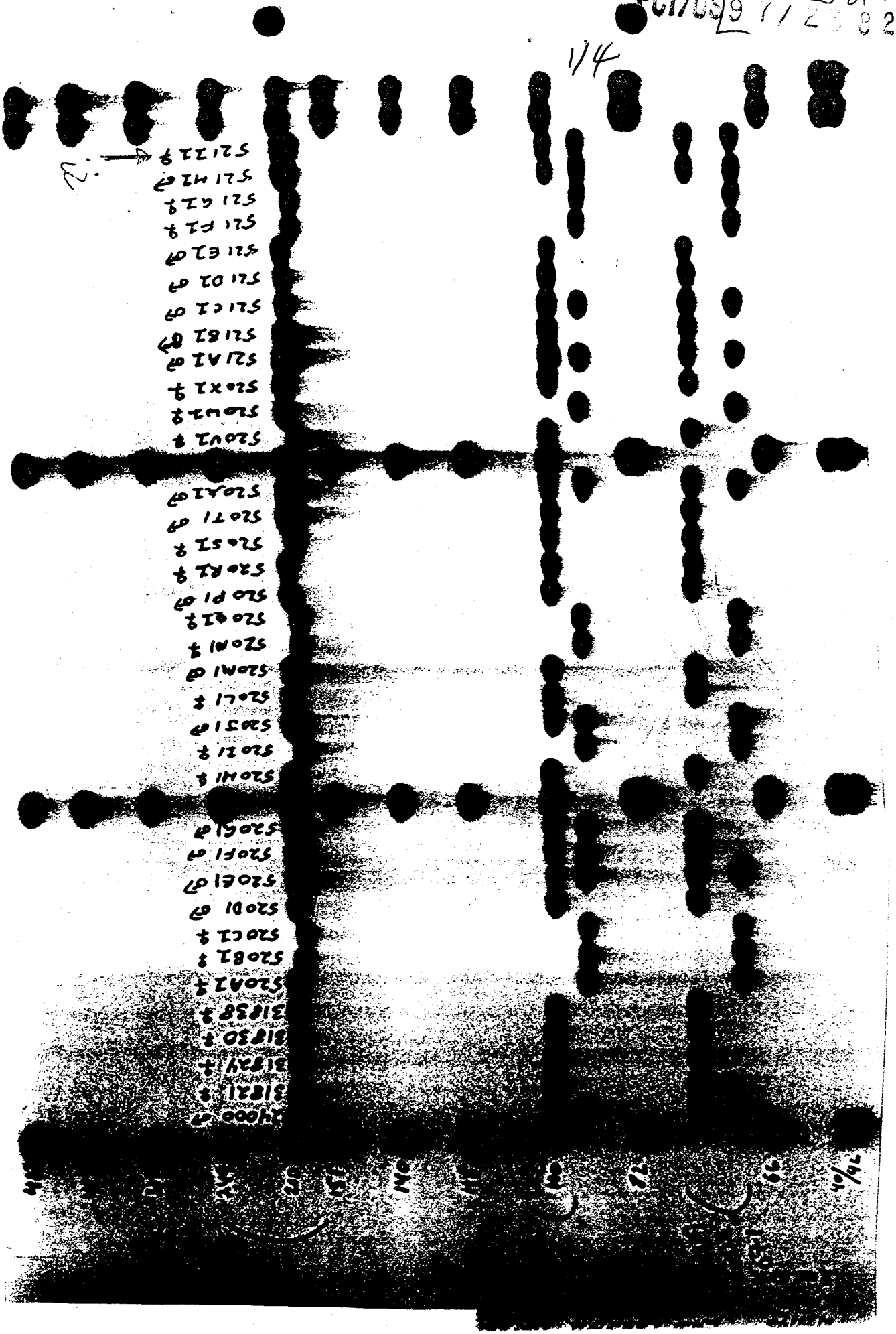

S21I2 ♀
S21M2 ♂
S21A1 ♀
S21F1 ♀
S21E2 ♂
S21D2 ♂
S21C1 ♂
S21B1 ♂
S21A1 ♂
S20X1 ♀
S20W1 ♀
S20V1 ♀
S20A1 ♂
S20T1 ♂
S20S1 ♀
S20R1 ♀
S20P1 ♂
S20Q1 ♀
S20M1 ♀
S20M1 ♂
S20L1 ♀
S20J1 ♂
S20I1 ♀
S20H1 ♀
S20G1 ♂
S20F1 ♂
S20E1 ♂
S20D1 ♂
S20C1 ♀
S20B1 ♀
S19A1 ♀
S18Z8 ♀
S18Z0 ♀
S18Z4 ♀
S18Z1 ♀
S18000 ♂

FIGURE 1

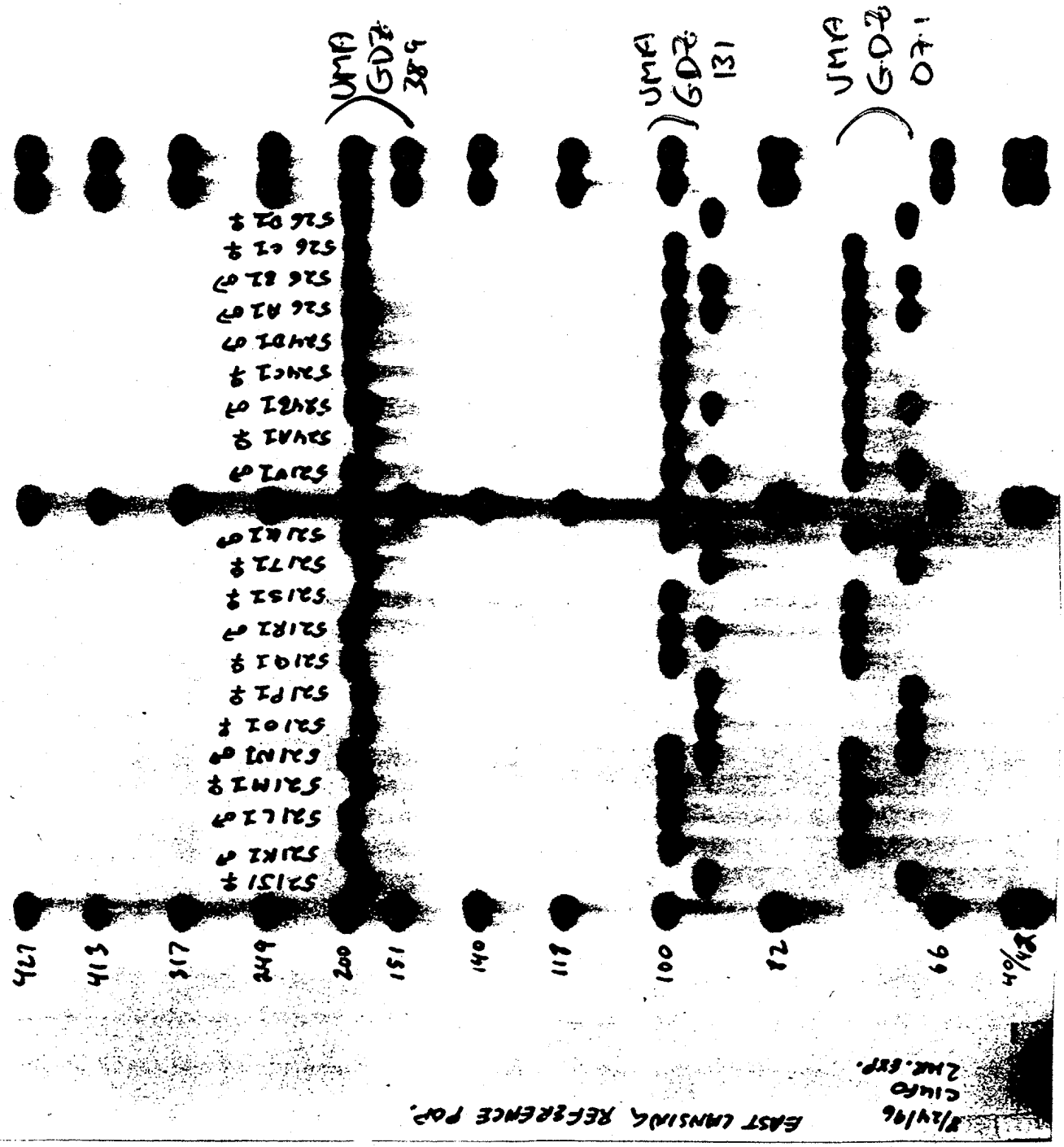
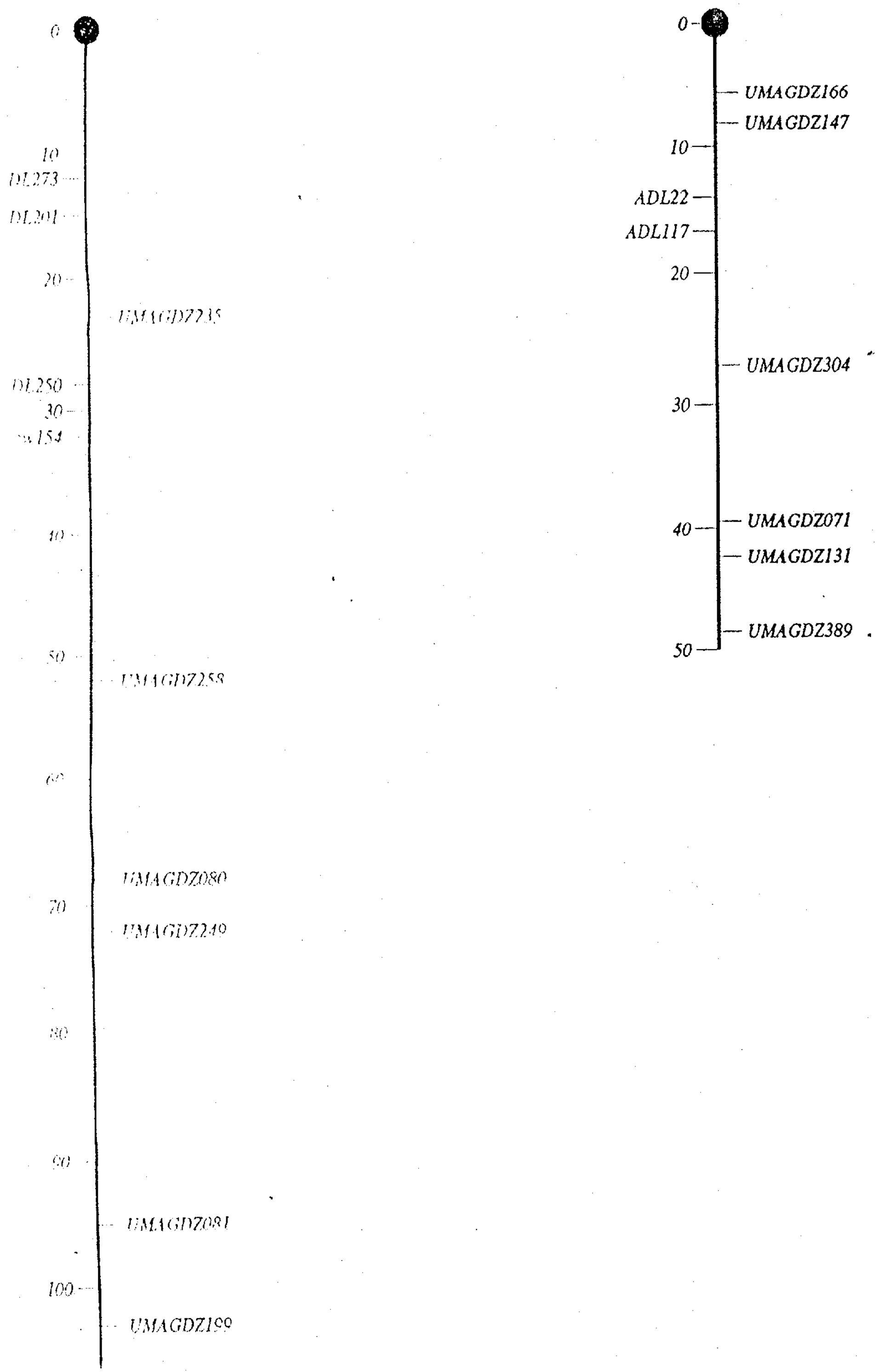


FIGURE 1 (Cont)

FIGURE 2


0
10
20
30
40
50
UMAGDZ166
UMAGDZ147
ADL22
ADL117
UMAGDZ304
UMAGDZ071
UMAGDZ131
UMAGDZ389
UMAGDZ258
UMAGDZ080
UMAGDZ081
100

Chicken Z Chromosome Microsatellites
Microsatellite composition

S. Ciufo

Clone	Repeat
UMGDZ043	$(AAC)_7$
UMGDZ071	$(CA)_5$
UMGDZ080	$(AC)_{16}$
UMGDZ081	$(CT)_{13}(AC)_{13}(CT)_7$
UMGDZ131	$(CA)_4$
UMGDZ147	$(CA)_{22}$
UMGDZ166	$(AC)_{15}$
UMGDZ196	$(AC)_{19}$
UMGDZ199	$(GT)_{12}$
UMGDZ204	$(AC)_{21}$
UMGDZ235	$(AC)_{15}$
UMGDZ249	$(AC)_{16}(TTC)_4$
UMGDZ258	$(TG)_{12}$
UMGDZ290	$(AC)_{23}$
UMGDZ304	$(AC)_{20}$
UMGDZ341	$(AC)_{22}$
UMGDZ398	$(CAA)_3$
UMGDZ420	$(GT)_{20}$
UMGDZ435	$(CA)_{11}$

FIGURE 3

SN 5210 06/29/99

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Date of mailing (*day/month/year*) 17 July 1998 (17.07.1998)	
Applicant's or agent's file reference 002076-001	**REPLY DUE** see paragraph 1 below
International application No. PCT/US98/08896	International filing date (*day/month/year*) 02 January 1998 (02.01.1998)
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International publication will now take place on 27 August 1998 (27.08.98).

Meanwhile, the International Bureau will communicate a copy of the international application to each designated Office, in accordance with PCT Article 20.

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The receiving Office (RO/US) is kindly requested to forward replacement sheets of drawings as well as complete addresses of the applicant/inventors AMBADY, Sakthikumar and SMYTH, J. Robert, Jr., if they have already been submitted by the applicant in response to form PCT/RO/106 dated 03 February 1998.

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NOTIFICATION OF RECEIPT OF RECORD COPY

(PCT Rule 24.2(a))

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Date of mailing (day/month/year)	**IMPORTANT NOTIFICATION**
16 July 1998 (16.07.98)	
Applicant's or agent's file reference	**International application No.**
002076-001	PCT/US98/08896

The applicant is hereby notified that the International Bureau has received the record copy of the international application as detailed below.

Name(s) of the applicant(s) and State(s) for which they are applicants:

UNIVERSITY OF MASSACHUSETTS (for all designated States except US)
PONCE DE LEON, F., Abel et al (for US)

International filing date	:	02 January 1998 (02.01.98)
Priority date(s) claimed	:	02 January 1997 (02.01.97)
Date of receipt of the record copy by the International Bureau	:	10 February 1998 (10.02.98)
List of designated Offices	:	

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ATTENTION

The applicant should carefully check the data appearing in this Notification. In case of any discrepancy between these data and the indications in the international application, the applicant should immediately inform the International Bureau.

In addition, the applicant's attention is drawn to the information contained in the Annex, relating to:

- [X] time limits for entry into the national phase
- [ ] confirmation of precautionary designations
- [ ] requirements regarding priority documents

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Date of mailing (*day/month/year*) 17 July 1998 (17.07.1998)	
Applicant's or agent's file reference 002076-001	**REPLY DUE** see paragraph 1 below
International application No. PCT/US98/08896	International filing date (*day/month/year*) 02 January 1998 (02.01.1998)
Applicant UNIVERSITY OF MASSACHUSETTS	

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Form PCT/IB/345 (July 1992)

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Date of mailing:
17 July 1998 (17.07.98)

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International application no.:	International publication no.:
PCT/US98/08896	

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1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer:

J. Zahra

Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

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INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
002076-001		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/US 98/ 08896	02/01/1998	02/01/1997
Applicant		
UNIVERSITY OF MASSACHUSETTS et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. [ ] **Certain claims were found unsearchable** (see Box I).

2. [ ] **Unity of invention is lacking** (see Box II).

3. [X] The international application contains disclosure of a **nucleotide and/or amino acid sequence listing** and the international search was carried out on the basis of the sequence listing
   - [ ] filed with the international application.
   - [X] furnished by the applicant separately from the international application,
     - [ ] but not accompanied by a statement to the effect that it did not include matter going beyond the disclosure in the international application as filed.
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4. With regard to the **title,**
   - [X] the text is approved as submitted by the applicant
   - [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**
   - [X] the text is approved as submitted by the applicant
   - [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this International Search Report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is:

   Figure No. _____
   - [ ] as suggested by the applicant.
   - [ ] because the applicant failed to suggest a figure.
   - [ ] because this figure better characterizes the invention.

   [X] None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/23822

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LEVIN I ET AL: "Genetic map of the chicken Z chromosome using random amplified polymorphic DNA (RAPD) markers." GENOMICS, (1993 APR) 16 (1) 224-30, XP002067078 ✓ cited in the application see the whole document	1-7
A	WO 94 07907 A (ZOOGEN INC) 14 April 1994 see the whole document	1-7
A	WO 96 39505 A (ISIS INNOVATION ;GRIFFITHS RICHARD (GB); TIWARI BELA (GB)) 12 December 1996 see the whole document	1-7

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 June 1998	18/06/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Molina Galan, E

1

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	ZIMMER R ET AL: "Generation of chicken Z - chromosome painting probes by microdissection for screening large-insert genomic libraries." CYTOGENETICS AND CELL GENETICS, (1997) 78 (2) 124-30, XP002067082 see the whole document ---	1-7
P,X	BIOLOGICAL ABSTRACTS, vol. 97, Philadelphia, PA, US; abstract no. 487182, PONCE DE LEON F A ET AL: "Chicken genome project: Chromosome-specific libraries and applications of genome scans to assess genomic variation." XP002067085 see abstract & REVISTA BRASILEIRA DE REPRODUCAO ANIMAL 21 (3). 1997. 102-105. ISSN: 0102-0803, -----	1-7

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	BRUFORD M W ET AL: "Minisatellite DNA markers in the chicken genome. II. Isolation and characterization of minisatellite loci." ANIMAL GENETICS, (1994 DEC) 25 (6) 391-9, XP002067079	
A	BIOLOGICAL ABSTRACTS, vol. 95, Philadelphia, PA, US; abstract no. 423269, PONCE DE LEON F A ET AL: "Analysis of the chicken NM7659 T(Z;1) translocation with chromosome painting probes and GBP banding." XP002067083 & EIGHTY-FOURTH ANNUAL MEETING OF THE POULTRY SCIENCE ASSOCIATION, INC., EDMONTON, ALBERTA, CANADA, AUGUST 14-18, 1995. POULTRY SCIENCE 74 (SUPPL. 1). 1995. 9. ISSN: 0032-5791,	
A	BIOLOGICAL ABSTRACTS, vol. 95, Philadelphia, PA, US; abstract no. 423268, AMBADY S ET AL: "A Z - chromosome specific DNA library." XP002067084 & EIGHTY-FOURTH ANNUAL MEETING OF THE POULTRY SCIENCE ASSOCIATION, INC., EDMONTON, ALBERTA, CANADA, AUGUST 14-18, 1995. POULTRY SCIENCE 74 (SUPPL. 1). 1995. 8. ISSN: 0032-5791,	
A	PONCE DE LEÓN ET AL.: "Development of a bovine X chromosome linkage group and painting probes to asses cattle, sheep and goat X chromosome segment homologies" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 93, April 1996, WASHINGTON US, pages 3450-3454, XP002067080 cited in the application	
P,X	AMBADY S ET AL: "Development of a chicken Z - chromosome -specific DNA library." JOURNAL OF HEREDITY, (1997 MAY-JUN) 88 (3) 247-9, XP002067081 see the whole document	1-7

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Int[redacted]nal Application No
PCT/US 97/23822

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9407907 A	14-04-1994	CA 2124220 A	14-04-1994
		AU 662564 B	07-09-1995
		AU 2696092 A	26-04-1994
		EP 0623139 A	09-11-1994
WO 9639505 A	12-12-1996	AU 5906996 A	24-12-1996
		EP 0832218 A	01-04-1998

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12Q 1/68 | A1

(11) International Publication Number: **WO 98/37243**

(43) International Publication Date: 27 August 1998 (27.08.98)

(21) International Application Number: PCT/US98/08896

(22) International Filing Date: 2 January 1998 (02.01.98)

(30) Priority Data:
60/034,410 2 January 1997 (02.01.97) US

(71) Applicant *(for all designated States except US)*: UNIVERSITY OF MASSACHUSETTS, A PUBLIC INSTITUTION OF HIGHER EDUCATION OF THE COMMONWEALTH OF MASSACHUSSETS, as represented by ITS AMHERST CAMPUS [US/US]; Office of Vice Chancellor for Research at Amherst, Amherst, MA 01002 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: PONCE DE LEON, F., Abel [US/US]; 134 Wildflower Drive, Amherst, MA 10002 (US). CIUFO, Stacy [US/US]; 56 Chesterfield Road, Amherst, MA 01002 (US). ROBL, James [US/US]; 196 Old Enfield, Belchertown, MA 01007 (US). AMBADY, Sakthikumar [IN/IN]; Kerala State (IN). SMYTH, J., Robert, Jr. [US/US]; Amherst, MA 01002 (US).

(74) Agent: TESKIN, Robin, L.; Burns, Doane, Swecker & Mathis, L.L.P., P.O. Box 1404, Alexandria, VA 22313-1404 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: Z-CHROMOSOMAL MARKERS DERIVED FROM CHICKEN (GALLUS DOMESTICUS) AND USE THEREOF IN CHROMOSOMAL MAPPING

(57) Abstract

We have developed a chicken (*Gallus domesticus*) Z-chromosome-specific DNA library in a phage vector, by means of chromosome microisolation and microcloning. The chromosomal origin, specificity and purity was evaluated by fluorescent *in situ* hybridization (FISH) on chicken metaphases. Heterologous chromosome painting, using this Z-chromosome-specific probe on turkey (*Meleagris gallopavo*) metaphases identified its homologous Z-chromosome, under the same stringent conditions as that used in the chicken, indicating a high degree of Z-chromosome sequence homology among these two species. This chicken Z-chromosome library will facilitate the development of Z-chromosome-specific DNA markers that will be useful for genetic mapping in the domestic chicken and related avian species. The Z-chromosome-specific DNA probe will also be useful for studies pertaining to the sex chromosome evolution in avian species.

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CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

Z-CHROMOSOMAL MARKERS DERIVED FROM CHICKEN (GALLUS DOMESTICUS) AND USE THEREOF IN CHROMOSOMAL MAPPING

Field of the Invention

The invention relates to novel chromosomal markers derived from chicken and use thereof.

Background of the Invention

Livestock genome maps have progressed very rapidly in the past few years due to the availability of highly polymorphic DNA markers. But in many species, the maps are not dense enough to facilitate a thorough search for quantitative trait loci (QTLs). This is especially true in the case of the chicken. The chicken haploid karyotype consists of 39 chromosomes that are classified into two categories - the macrochromosomes and the microchromosomes. The largest five pairs of macrochromosomes and the Z-chromosome represent about 55 percent of the total DNA content of the chicken genome. The Z-chromosome covers about 210 cM of the estimated 2500 - 3,000 cM of the chicken genome map (Levin et al. *Genomics*, 16:224-230 (1993)).

Knowledge of the genetic composition of the chicken Z-chromosome is limited, in spite of the fact that this chromosome has the most detailed linkage map for this species, largely generated by classical linkage test analyses (Bitgood and Somes, *Poultry Breeding and Genetics*, 2nd Ed., Crawford RD, ed., Amsterdam: Elsevier, pp. 469-495 (1990)). To date, 19 known loci and 14 genetic markers consisting of 3 chicken middle repetitive sequence element (CRI) markers, 8 random amplified polymorphic DNA (RAPD) markers and 3 microsatellites have been assigned to the chicken Z-chromosome (Bitgood and Somes, (Id.) (1990); Saitoh et al, *Chrom. Res.*, 1: 239-251 (1993); Cheng et al, *Poultry Sci.*, 74: 1855-1874 (1995)).

The avian sex chromosome constitution differs from that of mammals because females are heterogametic (ZW) and males homogametic (ZZ). It has been observed from comparative linkage analyses that some of the sex linked

genes in mammals are autosomal in chicken, while some of the sex linked genes in chicken are autosomal in mammals (Bitgood and Somes, (Id.) (1990)). Accordingly, obtaining further information concerning the Z-chromosome of chickens would be beneficial in identifying sex-linked genes in chickens and related species.

Brief Description and Objects of the Invention

Thus, it is an object of the invention to identify novel chromosomal markers from the Z-chromosome of chicken. It is further an object of the invention to use such markers to construct a Z-chromosome specific DNA map and to use such chromosomal markers to identify Z-chromosome homologs in related avian species, e.g., turkey.

In order to develop a dense genetic map for chicken, it is important to generate a large number of polymorphic markers per chromosome (Cheng et al, *Poultry Sci.*, 741:1855-1874 (1995)). One way of achieving this goal is to develop chromosome-specific libraries. Chromosome flow-sorting has been the method of choice for the generation of chromosome-specific libraries in humans (Fuscoe et al, *Cytogenet Cell Genet*, 43:79-86 (1986)) and in swine (Langford et al, *Anim. Genet*, 24: 261-267 (1993)). Development of flow-sorted chromosomes is technically demanding and frequently yield preparations which have some degree of contamination with other chromosomes (Hozier and Davis, *Anal. Biochem*, 200: 205-127 (1992)).

A more effective and direct way of generating chromosome-specific DNA libraries is by chromosome microisolation and microcloning of the chromosome of interest. Chromosome specific libraries generated by chromosome microisolation have been used in swine (Ambady et al, (unpublished data)), cattle (Ponce de León et al, *Proc. Natl. Acad. Sci., USA*, (in press) 1996)), and chicken (Li et al, *Proc. of the 10th Eur. Colloq. on Cytogenetics of Domestic Animals*, Utrecht Univ., The Neth., p. 11, August 18-21 (1992)) genetic mapping studies in order to develop maps for particular chromosomes.

Generation of polymorphic markers from chromosome-specific libraries for all of the 8 pairs of the chicken macrochromosomes will enable saturation of about 55-70% of the chicken genome. Chromosome-specific DNA can also be used as heterologous chromosome painting probes in closely and distantly related species for comparative genome analysis, study of chromosomal evolution, and for identifying gross chromosomal abnormalities.

This application, in particular, provides a chicken Z-chromosome-specific DNA library, Z-chromosomal markers and use thereof as probes to identify the Z-chromosome homolog in related species, such as turkey.

Brief Description of the Figures

Figure 1 shows amplification of microsatellite markers by PCR and identification of polymorphisims.

Figure 2 shows a genetic map constructed using the identified microsatellite markers.

Figure 3 shows dinucleotide repeats present in the identified microsatellite markers.

Detailed Description of the Invention

Microisolation and microcloning:

Chicken metaphases were prepared from chicken fibroblast cultures following standard procedures, fixed briefly for 5 minutes each in 9:1, 5:1 and 3:1 methanol:acetic acid and dropped on clean coverslips. Chromosome microisolation and cloning was performed following the procedure described by Ponce de León et al (*Proc. Natl. Acad. Sci., USA* (in press) (1996)). Briefly, twelve copies of the chicken Z-chromosome were microisolated and transferred to clean siliconized coverslips. Proteinase-K digestion, phenol-chloroform extraction, *Sau*3AI (50U/μl, New England Biolabs) digestion and ligation to custom prepared *Sau*3AI adaptors were performed in a nanoliter drop. Ligation

products were digested with BglI enzyme (Promega, 10 units/μl) to cleave off the adaptor dimers that form during the ligation process.

The ligation product was PCR amplified and 10 μl of the amplified product was run on an agarose gel to determine the size of the amplified products. A 2 μl volume of this original amplification was labeled by PCR, using biotin-16-dUTP (Boehringer Mannheim). The purity, specificity and origin of the DNA fragments was determined by FISH on chicken metaphases following the procedure described by Ponce de León et al (*Proc. Natl. Acad. Sci. USA* (in press) (1996)). The remainder of the PCR product was digested with *Sau*3AI and passed through a Microcon 30 (Amicon Inc.) spin column to cleave and remove the flanking adaptors respectively.

In order to produce a chicken Z-chromosome-specific phage library, the digested DNA was cloned in a lambda ZAP Express vector (Stratagene) and packaged using Gigapack II Gold packaging extract (Stratagene). The library was amplified by plate lysate method following the manufacturer's protocol and stored at -70°C in 7% DMSO and 0.3% chloroform. Average size of library inserts was determined by PCR amplification of 30 randomly picked clones using the T3 and T7 priming sites flanking the insert.

Fluorescent in situ hybridizations

The Z-chromosome-specific DNA fragments were fluorescently labeled by PCR with biotin-16-dUTP (3:1 ratio of dTTP:biotin-16-dUTP) and passed through a Sephadex G-50 column to remove unincorporated nucleotides. The protocol described by Ponce de León (*Proc. Natl. Acad. Sci., USA* (in press) (1996)) was followed. Briefly, 200 nanograms of labeled Z-chromosome specific DNA was mixed with 6 μg of chicken competitor DNA (average size 200-400 bp) and 5.8 μg of salmon sperm DNA (average size 200-400 bp), precipitated and resuspended in 12 μl of hybridization buffer consisting of 50% deionized formamide, 1X SSC and 100% dextran sulphate to achieve a final DNA concentration of 1 μg/μl. The hybridization mix was denatured at 75°C for 5

minutes and reannealed at 37°C for 10 minutes and deposited on denatured (70% formamide, 2X SSC at 70°C for 2 minutes) chicken or turkey metaphases, mounted, sealed with rubber cement and incubated in a humidified chamber at 37°C for 18 to 20 hours. The slides were washed in 50% formamide/2X SSC at 42°C for 15 minutes and 0.1X SSC at 60°C for 15 minutes. Blocking was done using 2% blocking reagent (Boehringer Mannheim) and the signals were detected using avidin-FITC (5 μg/ml, Vector labs) in 1% blocking solution. Slides were washed in 4X SSC/0.1% Tween-20 for 15 minutes at 42°C, stained for 10 minutes in propidium iodide (400 ng/ml in 2X SSC) and rinsed for 5 minutes in 2X SSC/0.01% Tween-20. Slides were mounted in p-phenylenediamine-11 (PPD-11) antifade and observed under a Zeiss Axioskop fluorescent microscope.

Results

A chicken Z-chromosome specific DNA cocktail was developed by chromosome microisolation, *Sau*3AI digestion, adaptor ligation and PCR amplification. The amplified DNA fragments ranged in size from 400 bp to 1600 bp with the bulk of the DNA in the 500-1000 bp range. The origin, specificity and purity of the chromosomal DNA fragments was verified by FISH after PCR labeling of a small fraction of the DNA cocktail. The probes showed specific hybridization signal on a medium sized submetacentric chromosome identified as the Z-chromosome based on its morphology and G-banding pattern. After having confirmed the origin and purity of the preparation, the adaptors flanking the inserts were removed by *Sau*3AI digestion and column purification. Cloning was performed using equimolar ratios of the inserts to the vector ends (lambda ZAP Express, Stratagene). The original library consisted of a total of 8.48×10^5 plaques representing about 14 chicken Z-chromosome equivalents. The final titer of the amplified library was 1.2×10^{12} pfu/ml.

Thirty random plaques were selected and the inserts PCR-amplified using the T3/T7 priming sites flanking the inserts. The average insert size was about 1,000 bp (data not shown). This library was screened to identify microsatellite

containing clones to increase the marker density of the chicken Z-chromosome genetic linkage map.

Heterologous painting of turkey metaphase chromosomes:

The labeled chicken Z-chromosome-specific DNA fragments were used to perform FISH analysis on turkey metaphase chromosomes following the procedure described previously. Washes at the same stringency showed strong hybridization signals on a medium-sized submetacentric chromosome in turkey metaphases (data not shown). This chromosome was identified as the Z-chromosome homolog in the turkey. The obtained results indicate that the chicken and turkey Z-chromosome sequences are highly conserved. The red-legged partridge Z-chromosome has also been shown to be homologous to the chicken Z-chromosome (Dias el al, *Proc. of the XXIV Int. Cont. on Anim. Genet.*, Prague, Czech. p. 133 (July 23-24, 1994)). These results are similar to the FISH results obtained when the bovine X-chromosome painting probes were used on sheep and goat chromosomes (Ponce de León el al, *Proc. Natl. Acad. Sci., USA* (in press) (1996)) and with human X-chromosome probes on a wide range of mammalian species (Schertan el al, *Nat. Genet.*, 6:342-347 (1994)) indicating the high degree of sex chromosome conservation among all the mammalian species studied. Solinas-Toldo et al (*Genomics*, 27: 489-496 (1995)) have previously shown that human chromosome-specific painting probes could identify chromosomal segments in bovine that are homologous to specific human chromosomes. It is expected based on our results that chicken chromosome painting probes can similarly be used in closely and distantly related avian species to identify gross chromosomal rearrangements such as translocations and duplications that have occurred during avian evolution. Since the chicken Z-chromosome sequences are highly conserved in the turkey, the chicken Z-chromosome-specific microsatellite markers should be particularly useful for genetic mapping in turkey.

Conclusions

Genetic and physical mapping of human and animal genomes has been greatly facilitated by the use of chromosome specific DNA libraries. Mapping with libraries specific to a chromosome or chromosomal region increases marker saturation by reducing the gaps resulting from a purely random shotgun approach. This study was undertaken to construct a genetic and physical map of microsatellites on the chicken Z chromosome. This chromosome is the fifth largest in the chicken genome, comprising about 8% of the total. Notwithstanding its size, very few microsatellites have been assigned to it. DNA originating from the chicken Z chromosome was previously isolated and reported. This was used to construct a small insert library in Lambda ZAP Express, representing 14 chromosome equivalents. This library was screened for microsatellites with an (AC) 12 oligo, and positive clones were isolated. Confirmation of the presence of the microsatellite, as well as its approximate location along the cloned fragment was accomplished by PCR amplification. Clones with adequate flanking regions were sequenced, and primers for 19 microsatellites were constructed. These primers were used to genotype individuals from the East Lansing Poultry Reference Population and a linkage map was constructed. Fourteen markers were scorable and polymorphic in this population. The resulting map contains 12 markers in two linkage groups spanning 90 Cm and two unlinked markers. The physical location of each marker was established by fluorescent *in situ* hybridization (FISH). Preliminary results with four markers allowed the assignment of one linkage group to the long arm of the Z chromosome, and one to the short arm.

The following nucleic acid sequences are microsatellite markers identified by the above methods. As discussed supra, these markers are useful for genetic mapping and for study of the sex chromosome structure in avian species. Also, such markers should enable the identification of genes encoding desirable traits, e.g., genes involved in growth rates, and for identifying sex-linked genotypes.

EXAMPLE

The specific Gallus domesticus microsatellite markers identified are set forth below. As noted, these DNA markers will be useful for genetic mapping of domestic chicken as well as related avian species and for studies pertaining to evolution of the sex chromosome in avian species.

SEQUENCE 1 (43. Seq)

1 gatcactttc cctaatattc ttgtgtttct tgtttgttga cctgtaatgc

1 agttctgagt tttggaaagg aactaattaa gaccagagga gagataattt

101 tcttttatca aaaaacaaac aaacaaacaa aaaaacgaat tcttaccact

151 ttacaaaaat tttccatttt gaaggccagt acagccatag cattcatcta

201 ctttttgctt tggat

SEQUENCE 2 (71. Seq)

1 gatcaggtgg cctgtagtag acaacaacaa caatggggtg ccctttgttg

51 ccttagtctc taactcgcac ccacacacac tttcaagttg cttgtggcca

101 ttcttcaggg acagttcttc acaatctatt cctttcctga tgtagaaggc

151 gtcacctcct cccctcctgc ctcgtttgtc ccttctaaac tgcaggtatt

201 agtattgata gctaaggtca agtcatggga accatctcac caggtttcag

251 tgttggcaac tatgttatgc tttcttagga gcatggtggt tccaactctt

301 ccctgcttat ttcccaagct gtgtgtgatg gtaggatagc attcaagtgg

351 gaggagccta tcggcttttt ggaggtactc ctaaatccct gatattcccc

401 tgattcccgt acttcttcct tgccaagggc ccgccaatgc atagttcaat

451 ttctcatgca gacgctaagg aaaggtggac cc

SEQUENCE 3 (80 Seq.)

1 gatcgtatgt attttttac ataggataga aaatggccaa taggaaataa
51 gacagtacag ctactaagaa agaaacacaa ttacacacac acacacacac
101 acacacacac acacatttga aaaacgcgct gcacagcagt gtgggtattt
151 tttcacaaga gagacacact ctacagtaca cagccagctc tactttgtcg
201 cacagtctca gtgtgtgttt gccaacagga cgcggttcac agggagatat
251 tgtcctcttg tgtgtgtgga gacacagaga cagag

SEQUENCE 4 (81. Seq)

1 gatcccctgg aggaagggca atggcaaccc actccagtat tcttgcctga
51 agaataccat ggtcagtttt gcctcctggg ctatagtcca tggggttgca
101 aagagtcagg catgactgag cgactctctc tctctctctc tctctctctc
151 acacacacac acacacacac acacacggcg tctctctctc tctctataca
201 tataggctgt gtgtctcgct attctcacat gagggaaact catatctagc
251 acgtggcaca aatattgttt gtggctctca caaaagacat gtgggcgcac
301 aaaggtcccc ccccggtgga tacancgcct tggtttttta taacccaagc
351 ctgtg

SEQUENCE 5 (131 Seq)

1 gatcacatat gtaaactagg gaattgcata ataagattaa atgtaggtgt
51 agaacgtggc atgaaggaag gtagaattag gtggtaccta tctcttctga
101 aacaaactga gaatcctact accaatcaac atattctaca taccacacac
151 acatttttc tcgagtaaaa tataaactaa tgagaaactt ccctag

SEQUENCE 6 (147. Seq)

1 gatcccaagc aacacatagn cagacaatca cacacacaca cacacacaca
51 cacacacaca cacacacaca cacatcctct ccccacaata catcccgaga
101 ggggggagag acactctctc tccctctcta taggggagac ccggagagct
151 ggctctgttg tctctctaca ccggacatac agtggagcac atctcacact
201 tgtgtctttg tctctctaca ccggacatac agtggagcac atctcacact
251 tgtgtctcta tctctccctg tccctgttga tccatctctc ttcacacatc
301 tctccagatc ttagcgctag agtctcctgt cttctctctg cgcaatttgt
351 gtgatagaga cacctgatat gttgtgtggg ggagacatct gtgtgtctct
401 gtgtcatccc agaggatttt tctctcccac acttagaggc cttctcaaga
451 gatgggaggt tttaatgggg tgtg

SEQUENCE 7 (166. Seq)

1 gatcattctt ctgtttccca ttctaatggg aattctccac acacacacac
51 acacacacac acacacacat cttcttcccc ttacatggaa aaaaatcctc
101 cacacccctg gacactgatt actctccctc ttcccagaga gagatc

SEQUENCE 8 (196. Seq)

1 gatcccctag agaagggaat ggctactcac tccagtattc ttgcctggag
51 aattccgtgg tcagaggagc ctggaaggct ataatccata gagtcgcaag
101 agtcagacag gactgagtga ctaacacaca catgcacaca cacacacaca
151 cacacacaca cttgctctag ggagaggcat agagatgtaa tctctcctaa
201 aatgggggtg gcgatggccc ctgcggccaa gtaatcgcca cacatgcgta
251 ttccccttaa gattgggtta ggcctccctt atgaggagag accagggaga
301 gaatgggctc tctctctctc tcactcccca accgagtaag tggtaaaaaa
351 ggttttcctg gattacaatt ttggtgttac agaattggaa aaaaatattt
401 ttggggctcc cccctcagtt ta

SEQUENCE 9 (199. Seq)

1 ctagcaaaaa caccccccaca agttatgaaa acaacggctt aatatagtaa
51 tgtgtgtgtg tgtgtgtgtg tgttgcacac cacagttttc tctgatactc
101 aaacctctct ctttctctac aggggccccc cataacacag cggctgagat
151 gtgtgacggg aaggcgtggc cttttacaca tttgtggtat ggtctgccaa
201 ggcccccctat tgcccccccac aactacggag atacactagg ggcgacccgc
251 aggcgcgcga cccccaggtg gggccccgag

SEQUENCE 10 (204. Seq)

1 ctttaggagg ttctctcgag taagcttttt ggatttcttt ggttcccaag
51 catcacatgg tacaggcagt cacacacaca cacatacaca cacacacaca
101 cacacacaca cactcctctc cccacaatac ataccgagag gggggagaga
151 cactctctct ccctctctat agggggagcc ccacagagct ggctctgttg
201 tctctctcca ccggacatac agtggagcac atctcacact tctgtctcta
251 tctctccctg cccctgtgac atccatctct cttcacacaa tctcacccag
301 gatcttagcg ctagagaccc cctgtccttc ttctcctggg gaaatttttt
351 gtggataaga gacacccgat atattggtgt gggggagaac atcttgtgag
401 gtctctgttg tgccatccca acaggaattt ttatctcccc cacaattaga
451 ggcccctcct caagagtgtg tgagggtt

SEQUENCE 11 (235. Seq)

1 gatcacagat gtatgtattt ttttacatag gatagaaaat ggacaatagg
51 aaataagaca gtacagctac taagaaagaa cccacattta cacacacaca
101 cacacacaca cacacacaca agtgtttaat ccgctgcaca gcattgtgga
151 catttttaca caagagagac acactctaca gtttgcgccc agctctag

SEQUENCE 12 (249. Seq.)

1 gatcattctt ctgtttccca ttctaatgga attctccaca cacacacaca
51 cacacacaca cacacactct tctttctcct gacatggaaa aatctccccc
101 acaccccggg acactgattt ctctccctct ccccaacact gtgagcaaga
151 ggagtttatt ttgtgtgtgt cactcttcca gggagagaga gatc

SEQUENCE 13 (258. Seq)

1 ctaggcatcg gttgggaggt ggtgagtaat tacttgtctg acattagtcc
51 tgtaacattg ggtgtgtgtg tgtgtgtgtg tgtgtattcc ccttgggaat
101 tggttttctc aaccacaagt tcttcttttt ttttttctc ccccctttc
151 ttctgaaaat aagtacttgg ggggtttccg ccccccccgg taaataaaat

SEQUENCE 14 (290. Seq)

1 ctagtggctc ccaagcaaca catagccaga caacacacac acacacacac
51 acacacacac acacacacac acacacactc ctctccccac aatacatccc
101 gagaggggggg agagacactc tctctccctc tctatagcgg gagccccaca
151 gagctggctc tgctgtctct ctacaccgga catacagtgg agcacatctc
201 acattcgtgt ctctatctct ccctgccccc ggtgacatac atctctcttc
251 acacatctca ccaggtctga gcgctagagt ctcctgtctt ctctctgcgc
301 aatatttgtg atagagacat ctgatatatt gtgtgtggga gacatcttgt
351 gagtctctgt gtgcatccca gaggattttt atctccccac actag

SEQUENCE 15 (309. Seq)

1 gatccatgaa aactttccga gttgtattgt ctaggtgaaa acacacacaa
51 acacacacac acacacacac acacaacagg gagatgagtc ttgcaagaga
101 ataggggaga gttatgtcac caagtctggt gaggtatata gcgtataggg
151 agccaacatg tcagacatct gatgtgctaa gattaacatt ttatttttatt
201 taatgtgtga gatctcatat agcggctctt cttatatatg acgtctcgca
251 atgtctcttt atgtgtgtta ttctctgagc ccctgggaga tatctgtcat
301 cagagagaag agacatacac atacaggggt tatatatttt ctccctgtgt
351 gtggagatgg agggtatttt ggacaagctc aacactcatt ggctcccaga
401 gagagaaaag gagcaactgt tgcacccggg gctctgtagc tgggatc

SEQUENCE 16 (341. Seq)

1 caattgggta catctacctg gtaccccacc cgggtggaaa atcgcatggg
51 cccgcggcgg ttctaggaag tactctcgag aagcttttgg gttctttggg
101 tcccaagcag cacatggaca ggcaatcaca cacacacaca cacacacaca
151 cacacacaca cacacacaca ctcctctccc cacaatacat cccgagaggg
201 gggagagtca ctctctctcc ctctctatag ggggcgcccc taagagctgg
251 ctctgttgtc tatctacacc gcacatacaa tggagcacaa ctcacactag

SEQUENCE 17 (398. Seq)

1 gatcaaagca tggaggtcat gccaggcact gaacaaaatg gtagagagtg
51 attctatgac tgactaagac ctcatgcaac aacaagtgaa gagtcacaac
101 tgcaaacaga agtacaactt agcaaatcct attttcagga aacactaaac
151 cgtaatactt gcacgatttt ttctttaata cagtaataat tcttttagaa
201 tttggatata tcttttaaga tacatatttg tctaaatacc aaggcaggat
251 atgagcataa aatagctaag gttagctatg gtgttatatt taagaagacc
301 acagagcaat aggagcatac ttttcttggg gtagaagggg cccttaaagg
351 tcacctag

SEQUENCE 18 (420. Seq)

1 ctagccacat cctataactc cactccacct ttaatcctga tttctgtgtc
51 tcttctctaa cctctatggc ctttctctaa agttccccaa tatcaacaat
101 ccttttcccc actgggacct ccagtttatt gattctacca tgtcactatc
151 catggtcaac cacttgtggt attataggat gtcgcgtgtg tgtgtgtgtg
201 tgtgtgcatg tgtgtgtgct tgggtgtcag agagttccaa tctgggggac
251 ctatggtttg taaacaacag gtctcttgcc aaggaagat

SEQUENCE 19 (435. Seq)

1 ctagcgctcg tgcccctgca gttcgacact cagtggctcc tccacacaca
51 cacacacaca cacatcaata tatatataga tagatagata gatagaggag
101 caatataagt ggcttctcta tttccagcat gttttgaaga gcataaactc
151 aacagagtat atataaatct gatgtgaccc atgtcatctg ctacagcatg
201 agaggggtta gtgatc

CLAIMS:

1. A Z-chromosomal marker DNA selected from the group consisting of Sequence I (43. Seq), Sequence 2 (71. Seq), Sequence 3 (80. Seq), Sequence 4 (81. Seq), Sequence 5 (131. Seq), Sequence 6 (147. Seq), Sequence 7 (166. Seq), Sequence 8 (196. Seq), Sequence 9 (199. Seq), Sequence 10 (204. Seq), Sequence 11 (235. Seq), Sequence 12 (249. Seq), Sequence 13 (258. Seq), Sequence 14 (290. Seq), Sequence 15 (309. Seq), Sequence 16 (341. Seq), Sequence 17 (398. Seq), Sequence 18 (420. Seq), and Sequence 19 (435. Seq).

2. A Z-chromosomal DNA library that contains at least one DNA sequence according to Claim 1.

3. A method of using at least one Z-chromosomal DNA according to Claim 1 for genetic mapping.

4. The method of Claim 3, wherein the genetic mapping is effected to construct a Z-chromosome specific DNA map.

5. The method of Claim 3, wherein the Z-chromosome DNA map is that of an avian species selected from the group consisting of chicken, turkey, partridge, duck, guinea hen, and goose.

6. The method of Claim 4, which is used to identify gross chromosomal rearrangements.

7. The method of Claim 6, wherein said chromosomal rearrangement comprises a translocation, deletion or duplication.

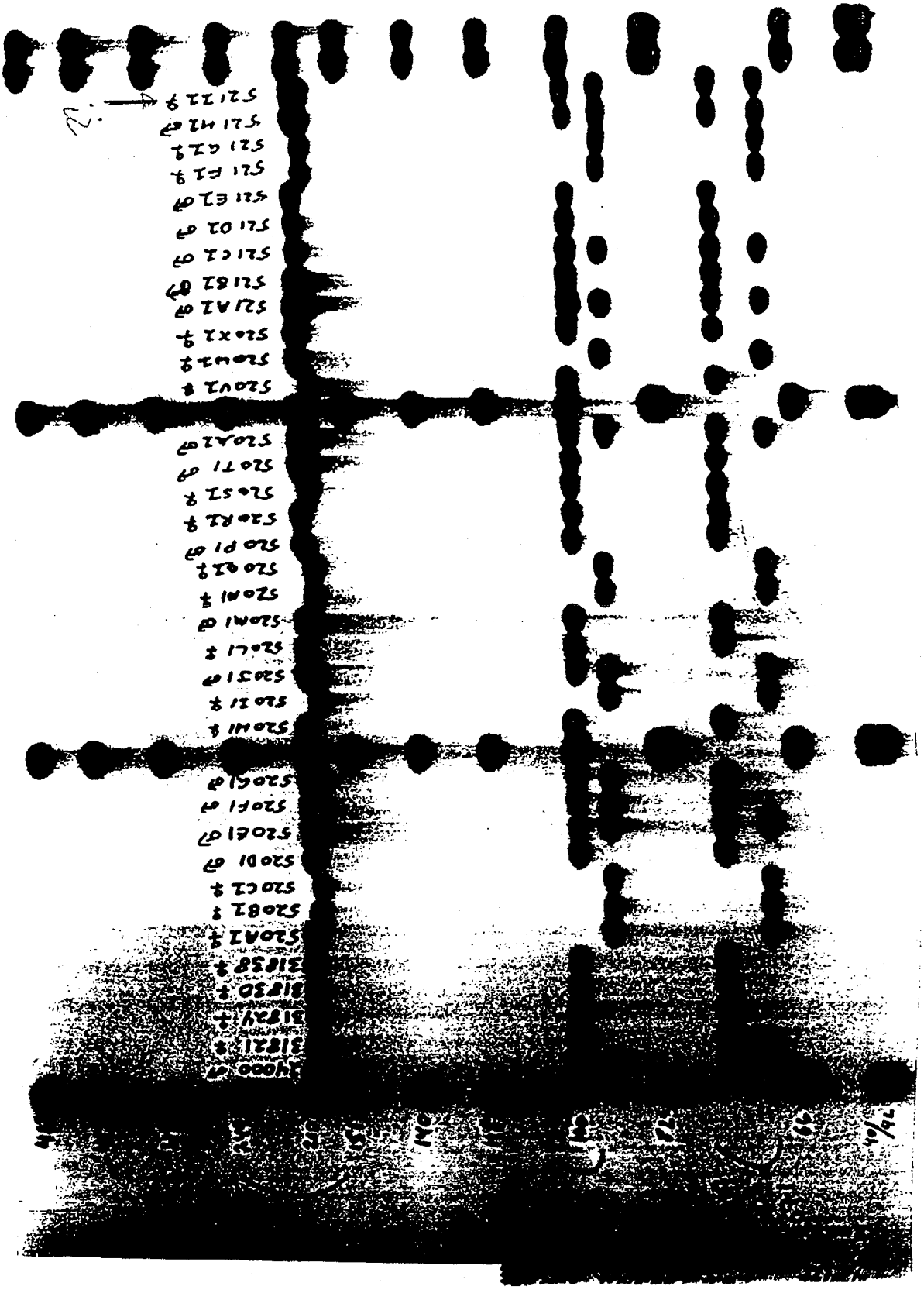


FIGURE 1

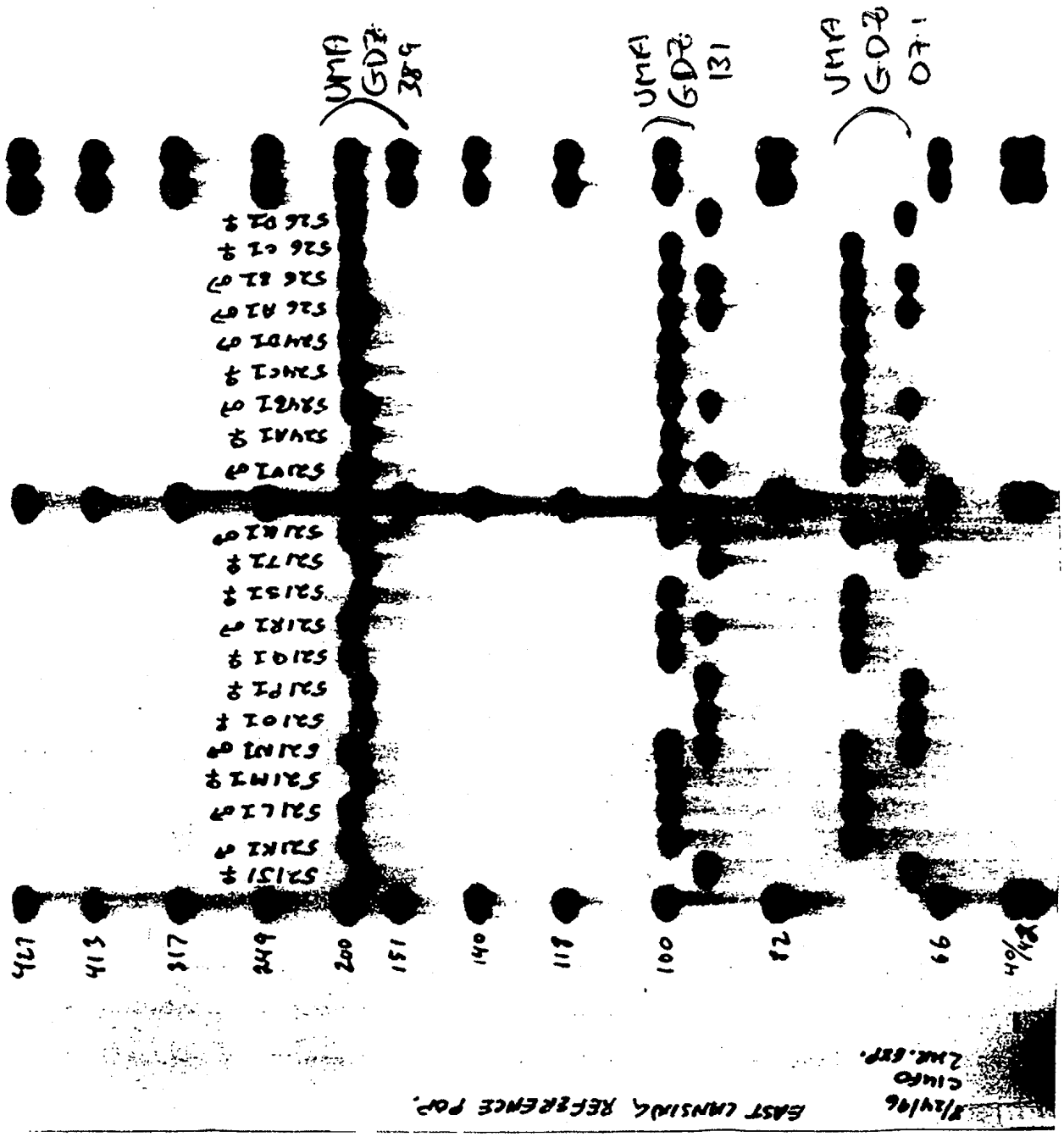
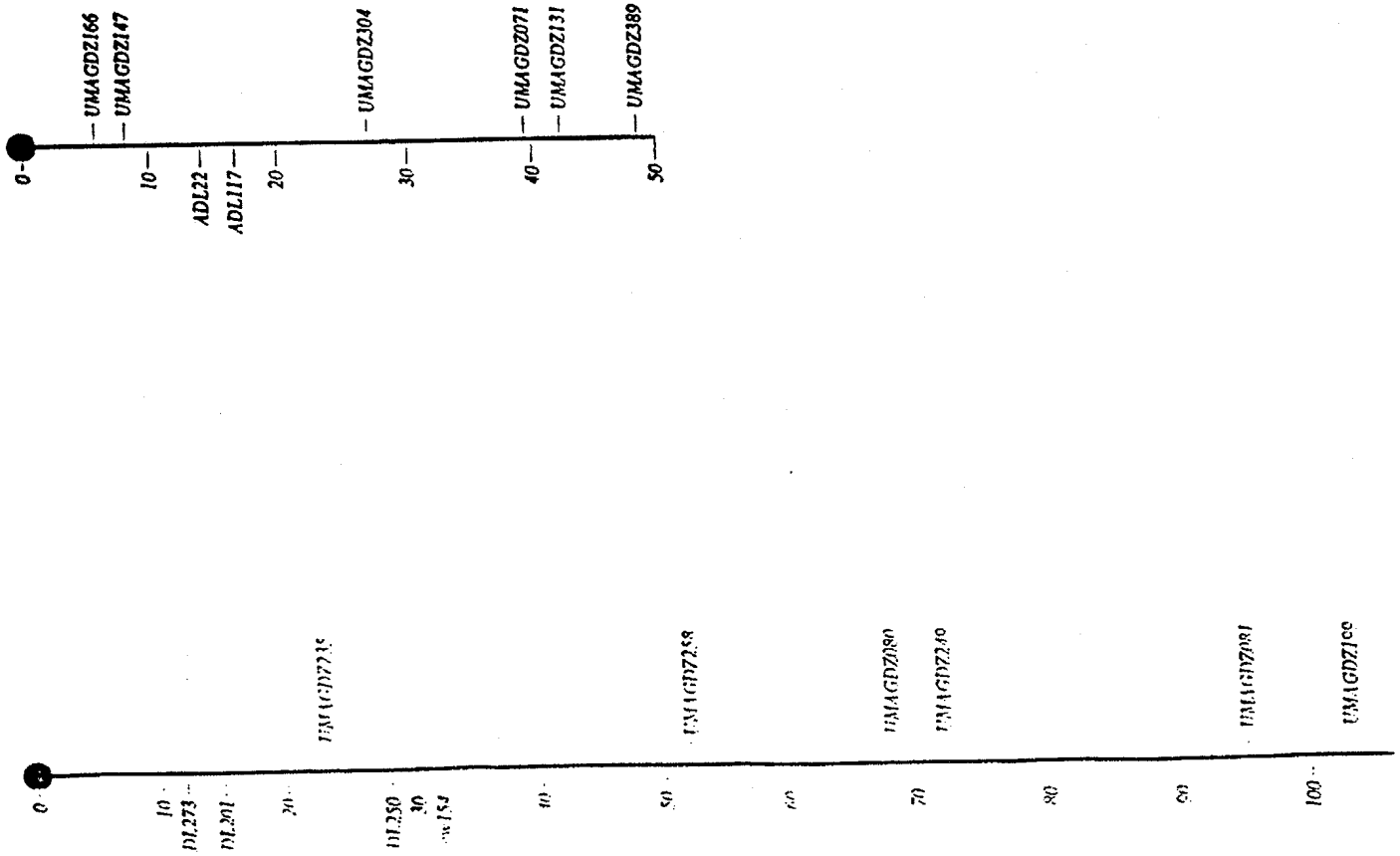


FIGURE 1 (Cont)

FIGURE 2


UMAGDZ166
UMAGDZ147
UMAGDZ304
UMAGDZ071
UMAGDZ131
UMAGDZ389
0
10
ADL22
ADLI17
20
30
40
50
60
70
80
90
100

Chicken Z Chromosome Microsatellites
Microsatellite composition

S. Ciufo

Clone	Repeat
UMGDZ043	$(AAC)_7$
UMGDZ071	$(CA)_5$
UMGDZ080	$(AC)_{16}$
UMGDZ081	$(CT)_{13}(AC)_{13}(CT)_7$
UMGDZ131	$(CA)_4$
UMGDZ147	$(CA)_{22}$
UMGDZ166	$(AC)_{15}$
UMGDZ196	$(AC)_{19}$
UMGDZ199	$(GT)_{12}$
UMGDZ204	$(AC)_{21}$
UMGDZ235	$(AC)_{15}$
UMGDZ249	$(AC)_{16}(TTC)_4$
UMGDZ258	$(TG)_{12}$
UMGDZ290	$(AC)_{23}$
UMGDZ304	$(AC)_{20}$
UMGDZ341	$(AC)_{22}$
UMGDZ398	$(CAA)_3$
UMGDZ420	$(GT)_{20}$
UMGDZ435	$(CA)_{11}$

FIGURE 3

INTERNATIONAL SEARCH REPORT

Int. ional Application No
PCT/US 98/08896

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LEVIN I ET AL: "Genetic map of the chicken Z chromosome using random amplified polymorphic DNA (RAPD) markers." GENOMICS, (1993 APR) 16 (1) 224-30, XP002067078 cited in the application see the whole document ---	1-7
A	WO 94 07907 A (ZOOGEN INC) 14 April 1994 see the whole document ---	1-7
A	WO 96 39505 A (ISIS INNOVATION ;GRIFFITHS RICHARD (GB); TIWARI BELA (GB)) 12 December 1996 see the whole document ---	1-7

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 June 1998	18/06/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Molina Galan, E

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BRUFORD M W ET AL: "Minisatellite DNA markers in the chicken genome. II. Isolation and characterization of minisatellite loci." ANIMAL GENETICS, (1994 DEC) 25 (6) 391-9, XP002067079 ---	
A	BIOLOGICAL ABSTRACTS, vol. 95, Philadelphia, PA, US; abstract no. 423269, PONCE DE LEON F A ET AL: "Analysis of the chicken NM7659 T(Z;1) translocation with chromosome painting probes and GBP banding." XP002067083 & EIGHTY-FOURTH ANNUAL MEETING OF THE POULTRY SCIENCE ASSOCIATION, INC., EDMONTON, ALBERTA, CANADA, AUGUST 14-18, 1995. POULTRY SCIENCE 74 (SUPPL. 1). 1995. 9. ISSN: 0032-5791, ---	
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A	PONCE DE LEÓN ET AL.: "Development of a bovine X chromosome linkage group and painting probes to asses cattle, sheep and goat X chromosome segment homologies" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 93, April 1996, WASHINGTON US, pages 3450-3454, XP002067080 cited in the application ---	
P,X	AMBADY S ET AL: "Development of a chicken Z - chromosome -specific DNA library." JOURNAL OF HEREDITY, (1997 MAY-JUN) 88 (3) 247-9, XP002067081 see the whole document ---	1-7

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INTERNATIONAL SEARCH REPORT

Int ational Application No
PCT/US 98/08896

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	ZIMMER R ET AL: "Generation of chicken Z - chromosome painting probes by microdissection for screening large-insert genomic libraries." CYTOGENETICS AND CELL GENETICS, (1997) 78 (2) 124-30. XP002067082 see the whole document	1-7
P,X	BIOLOGICAL ABSTRACTS, vol. 97, Philadelphia, PA, US; abstract no. 487182, PONCE DE LEON F A ET AL: "Chicken genome project: Chromosome-specific libraries and applications of genome scans to assess genomic variation." XP002067085 see abstract & REVISTA BRASILEIRA DE REPRODUCAO ANIMAL 21 (3). 1997. 102-105. ISSN: 0102-0803.	1-7

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 98/08896

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		AU 662564 B	07-09-1995
		AU 2696092 A	26-04-1994
		EP 0623139 A	09-11-1994
WO 9639505 A	12-12-1996	AU 5906996 A	24-12-1996
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